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(71) Applicant *(for all designated States except US)*: THE RESEARCH AND DEVELOPMENT INSTITUTE, INC. [US/US]; 1711 West College, Bozeman, MT 59715 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: PYLE, Barry, H. [NZ/US]; 4985 Foster Lane, Belgrade, MT 59714 (US). MCFETERS, Gordon, A. [US/US]; 1320 Cherry Drive, Bozeman, MT 59715 (US). JUTILA, John, W. [US/US]; 516 South Grand, Bozeman, MT 59715 (US). SCHIEMANN, Donald, A. [US/US]; 10B Gallatin Drive, Bozeman, MT 59715 (US). BARGATZE, Robert, F. [US/US]; 1302 Wild Flower Way, Bozeman, MT 59715 (US). JUTILA, Mark, A. [US/US]; 3308 Sundance Drive, Bozeman, MT 59715 (US).

(74) Agents: PRICE, Robert, L. et al.; Lowe, Price, LeBlanc & Becker, Suite 300, 99 Canal Center Plaza, Alexandria, VA 22314 (US).

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(57) Abstract

A rapid method for the detection, identification and enumeration of specific respiring microorganisms. The method includes steps of a) passing a microbial sample through a collecting device to capture the cells; b) adding to the collecting device a fluorochrome dye specific for the detection of respiring cells and allowing the dye to incubate; c) treating the collecting device with a reactive fluorescent antibody which reacts with a target microorganism of interest present in said microbial sample; d) mounting the collecting device for examination by fluorescence microscopy in which a suitable light system is used to excite the fluorochrome dye and fluorescent antibody to fluoresce; and e) quantifying the respiring cells. Alternative embodiments include the use of immunomagnetic beads and other means of cell capture, and employing fluorescent oligonucleotide probes rather than fluorescent antibodies.

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SIMPLE, RAPID METHOD FOR THE DETECTION, IDENTIFICATION AND ENUMERATION OF SPECIFIC VIABLE MICROORGANISMS

Technical Field

The present invention relates to the field of methods of detection, identification and enumeration of respiring microorganisms.

Background

The detection, identification and enumeration of individual respiring bacteria or other microorganisms is important to and widely used in biological research, clinical microbiology, cancer diagnosis and treatment, environmental science, food safety, toxicology, and research and development in basic and applied biology.

Specifically, in public health and environmental microbiology, there is a need for rapid methods to enumerate and identify specific viable bacteria and other microbes. In foods, robust, reliable testing methods are needed that provide results more rapidly than conventional methods, with similar or greater sensitivity and specificity. This requirement is also of particular concern in relation to water which is reclaimed for potable use, such as on the U.S. Space Station.

Food-borne illnesses caused by bacterial pathogens represent a significant cause of morbidity, mortality and economic loss in the U.S. Recent estimates by the U.S.D.A. suggest that there are 3.6 to 7.1 million food-borne disease cases each year contributing to medical costs and productivity losses of $2.9 to $6.7 billion(3).

In recent years, there has been a dramatic increase in the number of cases of illness caused by the food-borne bacterium E. coli (O157:H7) (4,5). Since

1982, when the new pathotype of E. coli (VTEC) was identified in Oregon and Michigan, there have been hundreds of confirmed cases of severe hemorrhagic colitis caused by VTEC. This includes hemolytic uremic syndrome, a long term debilitating and fatal illness, especially in children. The seriousness of this public health problem was made apparent in 1992-1993 when over 500 cases were identified as having derived from the consumption of ground beef in many western states.

Cultural methods have been used to detect and enumerate bacteria in environmental samples for over a century. However, for almost 50 years it has been known that these methods yield only a small percentage of the actual population in a sample (54). Early explanations for the discrepancies between culture and direct counting methods included aggregation of bacteria into clumps (18,53), and failure to form colonies on plating media because of particular nutritional requirements (17). Other inadequacies of traditional plate counting methods, including cell injury and the viable but non- culturable state, have been discussed by several investigators (e.g., 14,16,18,25,30,31,39). While membrane filter techniques permit concentration of bacteria and their separation from inhibitory materials in the sample, they suffer from the drawbacks inherent in plating methods because of their reliance on colony formation.

In the older most probable number (MPN) (29) and more recent presence-absence (P-A) (7) approaches, liquid culture media are employed which may facilitate improved detection of some bacteria compared to plating methods. These methods have, however, been criticized for their lack of precision and possible biases (1). In recent years, media have been developed for the simultaneous detection and confirmation of coliforms and Escherichia coli (e.g., 1,13,26). While these systems offer advantages over conventional liquid media, especially in

terms of the time required to obtain a confirmed result, there are persistent statistical concerns relating to the MPN and P-A techniques on which they are based, such as their wide confidence intervals and biases.

There is also a dramatic increase in the number of cases of food-borne *Escherichia coli* in recent years and the prevalence of meat samples which are contaminated with these organisms suggest a need for rapid, specific bacterial detection methods. Conventional methods may grossly underestimate the numbers of specific target bacteria in a sample. One of the obstacles to the development and application of rapid, direct methods for the detection of specific pathogenic bacteria in foods is the need to concentrate the target bacteria and separate them from suspensions of the product. It is normally required that a contaminant should be detectable at less than 1 colony forming unit per 25 g sample. Filtration is usually avoided because it is not possible to filter large samples of meat homogenate even after large particles have been allowed to settle out.

The need for such a method and problems associated with known methods for detection, identification and enumeration of viable bacteria are set forth in Weaver et al., U.S. Patent No. 4,959,301, incorporated herein by reference. Present methods for enumeration of viable microorganisms are slow and generally labor intensive, and many newer methods which purport to give an enumeration are not based on actual viable cell counts.

Instead, many of these methods measure some average property of a large number of cells which, under well defined conditions, correlates with a count, but which under other conditions generally does not correlate accurately with a viable count.

Present cell analysis methods involve two major classes of assays. The first class rapidly detects and identifies specific cells directly from a primary

sample, but does not determine cell viability. The most widely used in this class are specific ligand binding assays, e.g. immunoassays and genetic probes. However, they require many cells, and do not distinguish between dead and viable cells. This restricts their use to samples in which sufficient numbers of cells are present, and to determinations in which direct assessment of the physiological state of the cell is irrelevant.

The second class of assays is used for viable cell determinations either directly using the primary sample, or using a subculture of the primary sample. The most traditional and widely used method is the plate count, which allows determination of single cell viability, based on growth, under many test conditions (see, for example, Hattori The Viable Count: Quantitative and Environmental Aspects, Brock/Springer, Madison, 1988). An important attribute of viable plate enumeration is that the time required to obtain a determination is independent of the concentration of the cell in the sample, as formation of each colony proceeds from an initial single cell. The major disadvantage is its slowness, as typical determinations require one-half to several days, and are also labor- and materials-intensive.

The disadvantages of viable plating can better be appreciated by drawing attention to its basic attributes. Viable plating is a well established, important method for qualitatively determining the growth of cells, particularly the presence or absence of growth for given conditions, and is often based on the growth of initial cells into distinct colonies. Viable plating typically involves the spreading of a suspension of cells onto the surface of a gel-containing petri dish, with or without the pouring of a gel layer over the first gel surface.

The gels are provided with nutrients, such that following an incubation period at a suitable temperature,

many generations of growth occur, which leads to formation of visible colonies. For many microorganisms formation of visible colonies requires growth for 22 to 30 generations and therefore produces colonies containing 10^7 to 10^9 cells. (See Sharpe, in Mechanizing Microbiology, A. N. Sharpe and D. S. Clark (Eds.) Charles C. Thomas, Springfield, 19-40, 1978). Although conventional viable plating leads to formation of colonies, and thereby provides a basis for counting viable cells by counting colonies, the presence or absence of colonies only allows an inference that the conditions present in the gel do or do not support growth. For this reason, conventional viable plating is not well suited to quantitative determinations such as cell growth rate and lag time, because viable plating based on visual inspection counts the number of colonies formed, but does not determine how the cellular material or amount of cellular constituents in the colonies varies with time.

An additional complication arises because the nutrient and metabolite concentrations within a colony comprise a microenvironment, which generally changes with time in a variable way as microcolonies increase to form larger colonies with many cells in close proximity. The microenvironment within a large colony can also have significant heterogeneity of chemical composition within the microcolony, so that different cells within a large colony experience different growth conditions. Further, although some methods are based on a straightforward extension and application of scanning optical methods for determination of optical properties of colonies on or in gel slabs, such methods suffer from relatively large cost, and, because of the relatively large gel slab size, does not allow incubation conditions to be changed rapidly at the site of the cells within the gel. (See Glaser in New Approaches to the Identification of

Microorganisms Proceedings of a Symposium on Rapid Methods and Automation in Microbiology, C. G. Heden and T. Illeni (Eds.), Wiley, N.Y., 3-12, 1975).

There are other limitations of plating techniques which may lead to underestimation of bacterial numbers. These include the possibility of bacterial clumping or attachment to particles in the sample. When this occurs and cells are not disaggregated or dislodged from particles, the assumption that a colony arises from a single cell is invalidated. Another consideration is the injury of bacteria by a variety of stressors which occur in water (McFeters, G.A. 1990. Enumeration, occurrence, and significance of injured indicator bacteria in drinking water. in Drinking Water Microbiology, McFeters (ed.), Springer-Verlag, New York, pp. 478-492) and food (Ray, B. (Ed.) 1989. Injured Index and Pathogenic Bacteri: Occurrence and Detection in Foods, Water and Feeds. CRC Press, Boca Raton).

Instrumented methods for rapidly determining cell or culture growth and/or metabolic activity have been developed which only partially address the limitations of the viable plate assay. These include optical techniques for growth determination such as those which measure the change in light scattering due to many cells in a liquid suspended culture (See, for example, Edberg and Berger, in Rapid Methods and Automation in Microbiology and Immunology, K. O. Habermehl, Ed., Springer-Verlag, Berlin, 215-221, 1985), and a variety of metabolic activity based techniques which measure changes due to many cells in an analyzed sample. Examples include changes in extracellular pH, electrical impedance, or fluorescence.

A disadvantage of all such metabolic activity methods is that they are based on combined effects of a large number of cells, and therefore generally require an initial process, based on plating, to obtain initial

colonies for purposes of inoculation of the analyzed sample, such that the determinations based on many cells at least are based on a monopopulation, i.e. a population comprised nominally of the same type of cells. For this reason, although a total population cell determination may itself be rapid, it is generally preceded by a viable plating method, or its equivalent, which is slow. Thus, the total analysis time, counted from receipt of a primary or non-plated sample to a cell growth determination, is the sum of both, and therefore still long.

Further, because such determinations are based on the combined effect of a large, but unknown number, of cells, such total population determinations do not actually yield a count. In contrast, determinations based on many individual measurements, each associated with an initial single cell, can yield a count.

Finally, because these total population methods are based on the combined effects of many cells, the time required for a determination becomes significantly longer as the number of cells decreases, i.e. as the sample's cell concentration decreases.

Similarly, prior use of flow cytometry for cell growth measurements (see, for example, Hadley et al. in Instrumental Methods for Rapid Microbiological Analysis, Nelson (Ed.), VCH, Weinheim, 67-89, 1985) is limited, because conventional use of flow cytometry performs measurements on individual cells, or clumps of cells which naturally adhere, in an aqueous liquid suspension, and therefore does not have the capability to measure colony formation. For this reason, prior use of flow cytometry can only measure total numbers of cells in a volume in order to determine average growth, and must also, therefore, involve a careful volume measurement, and is dependent on the signal-to-noise ratio of single cell measurements. This signal-to-noise ratio is less

than satisfactory for many measurements (see, for example, Shapiro Practical Flow Cytometry, R. Liss, New York, 1985; Hadley et al. in Instrumental Methods for Rapid Microbiological Analysis, Nelson (Ed.), VCH, Weinheim, 67-89, 1985).

Likewise, quantitative microscopy and image analysis combined with conventional gel preparations, such as gel slabs, petri dishes and the like, although capable of determining colony formation, is tedious, and in manual versions, conventional gel slabs, petri dishes and the like, cannot provide physical manipulability or a sufficiently fast (small) characteristic diffusion time within the gel, so that cells cannot be rapidly and conveniently exposed to different growth conditions, such as rapid changes in concentrations of nutrients, drugs, hormones, enzymes, antibodies and other chemicals. In addition, conventional gel slabs, petri dishes and the like cannot be readily manipulated physically because of their size, and therefore cannot be readily used for exposure of gel-entrapped cells to in vivo conditions.

Mansour et al., U.S. Patent No. 4,693,972 teaches a composition and method for rapid detection of microorganisms in clinical samples. The method for detection of microorganisms in a body fluid sample includes detecting a microorganism after treatment of the sample with a lysing agent in order to dissolve sample components other than microorganisms, and staining with a fluorescent dye.

Wolf et al., United States Patent No. 4,972,258 discloses a scanning laser microscope system and methods of use. The patent discloses a yeast culture was placed on a black polycarbonate filter. The filter was then overlaid with a fluorescent stain. The filter was rinsed in a succession of steps and then the filter was air dried and placed on top of a glass microscope slide. The fluorescent stain is an indicator of viability and direct

staining of microorganisms may be used for detection and enumeration and analysis of the microorganisms. The patent also discloses an indirect immunofluorescence assay in which a target microorganism is labelled with a primary non-conjugated antibody specific for microorganisms containing a target surface antigen. The microorganisms with bound antibody are fluorescently labelled using a fluorescein isothiocyanate conjugated secondary antibody which recognizes the primary antibody. Then the labelled microorganisms are placed on an appropriate surface and imaged using a scanning laser microscope. A filter is used which reflects the laser light and passes the fluorescence light. Wolf et al. does not disclose using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to detect respiring bacteria.

Weaver et al., United States Patent No. 4,959,301 teaches a process for rapidly enumerating viable entities. The patent describes the method of enumeration of viable biological entities which involves the determination of the number of viable biological entities per volume. The patent also discloses a method of determining the number of viable biological entities capable of growth per volume of a sample, comprising a) forming microdroplets of a volume of the sample wherein some but not all of the microdroplets contain viable biological entities; b) measuring the volumes of at least a portion of the microdroplets formed in step (a) to obtain the volumes of the microdroplets; (c) measuring the amount of biological material which constitutes part of the viable biological entities in the microdroplets and d) determining the statistical distribution of the biological material in the droplets and the volumes as indicative of the number of viable biological entities per volume of the sample. The measurement of the biological material accumulated within the microdroplets was enhanced after incubation with fluorescent stains.

The microdroplet volumes are measured optically and the optical means consist of light scattering, light absorbance, fluorescence, phosphorescence or chemiluminescence, and may also include fluorescence microscopy, light microscopy image analysis and video recording. Weaver et al. does not disclose using CTC to detect respiring bacteria.

Nader et al., U.S. Patent No. 5,173,187 discloses that by means of immunofluorescence, bacteria in a mixture can be specifically labelled, and individual types of bacteria can be detected qualitatively under a fluorescence microscope, with an image analysis system and then quantitatively in a flow cytometer. Nader et al. does not disclose using CTC to detect respiring bacteria.

Melnicoff et al., U.S. Patent No. 5,256,532 discloses methods, reagents and test kits for determining populations of biological entities. The patent discloses coupling of a detectable reporter substance non-selectively to the analyte which can be prokaryotic cells and later contacting the test sample with a specific binding substance which binds specifically to one characteristic determination of the analyte, and separating the components of the test sample and detecting the occurrence of the reporter substance in the sample. The reporter substance may be detected by fluorescence. In a preferred embodiment the non-selective reporter substance is linked to a lipid component of a biomembrane. Melnicoff et al. does not disclose using CTC to detect respiring bacteria.

Edberg et al., U.S. Patent No. 4,925,789 discloses a method and medium for use in detecting target microbes in a tube from a sample of contaminated material. The testing method of Egberg et al. provides a selective growth medium for a target microbe and includes a specific nutrient which only the target microbe can

metabolize. The specific nutrient is modified by attaching a sample altering moiety thereto, thereby converting the nutrient to a nutrient indicator. The sample altering moiety is activated to alter the sample only if the specific nutrient is metabolized by the target microbe. Edberg et al. does not disclose using CTC to detect respiring bacteria.

Waggoner et al. United States Patent No. 5,268,486 is directed to a water soluble luminescent dye for use in immunoassays and which can be used for the detection of bacteria.

Rodriguez, et al., Use of a Fluorescent Redox Probe for Direct Visualization of Actively Respiring Bacteria, *Applied and Environmental Microbiology*, June 1992, Vol 58, pages 1801-1808 discloses that a redox probe 5-cyano-23-ditolyl tetrazolium chloride (CTC) may be employed for direct epifluorescent microscopic enumeration of respiring bacteria in environmental samples. Rodriguez discloses the use of dark membrane filters (black Nuclepore membranes) for microscopic visualization of respiring bacteria. Rodriguez et al. does not disclose a method including a step of determining specificity of the bacteria using immunofluorescence.

Schaule et al., "Use of 5-Cyano-2,3-Ditolyl tetrazolium Chloride (CTC) for Quantifying Plant Sessile Respiring Bacteria in Drinking Water", *Applied and Environmental Microbiology*, November 1993, pages 3850-3857, Volume 59, Number 11, discloses that direct microscopic quantitation of respiring bacteria can be performed for drinking water samples using 5-Cyano-2,3-Ditolyl tetrazolium Chloride. Schaule et al. does not disclose a method including a step of determining specificity of the bacteria using immunofluorescence.

Chemical Abstracts Vol. 100 (1984) 188419d (U.S. Patent No. 4,434,236 to Freytag) discloses a method of rapid detection of analytes in a sample. The sample is

contacted with a solid phase having immobilized thereon an analyte analog to which there is displaceably bound a labeled anti-analyte antibody. The analyte may be a bacterium.

Chemical Abstracts Vol. 102 (1985) 42486t discloses a solid phase immunoassay for detecting bacteria. The immunoassay uses an immunoreactant attached to cells such as bacteria and a fluorescent or phosphorescent label.

However, prior art methods take a long time to conduct and do not simultaneously detect, identify and enumerate individual respiration of bacteria. The present method can be used to monitor the performance of water reclamation and storage systems. The present method may also be useful in tracing disease outbreaks, and in other public health situations such as water and wastewater treatment, storage and distribution. The method of the invention may also be used for routinely monitoring foods for quality control or grading purposes.

The present method overcomes the deficiencies of prior art methods by providing a rapid method for the detection, identification and enumeration of respiring microorganisms comprising the steps of

a) passing a microbial sample through a collecting device to capture the cells;

b) adding to the collecting device a fluorochrome dye specific for the detection of respiring microbes and allowing the dye to incubate;

c) treating the collecting device with a reactive fluorescent antibody which reacts with a target microorganism of interest present in said microbial sample;

d) mounting said collecting device for examination by fluorescence microscopy in which a suitable light filter system is used to excite the fluorochrome dye and fluorescent antibody to fluoresce; and

e) quantifying said respiring target microbial cells.

An advantage of the method of the present invention is that it is relatively rapid and minimizes actual labor input to about 1-2 hours with a total assay time of 3 to 6 hours. The procedure is amenable to automated examination using video image analysis technology.

The invention provides a rapid, reliable method to detect and enumerate viable verocytotoxic E. coli (VTEC) associated with hemorrhagic colitis in man.

Brief Description of the Drawings

Figure 1 shows a photomicrograph of cells of *Escherichia coli* 0157:H7 reacted with rabbit anti-0157 primary antiserum and with goat anti-rabbit fluorescein conjugate after incubation with CTC. Cells stained green have reacted with the fluorescein-labelled antibody. Those cells with an orange-yellow spot in them have reduced CTC to CTC formazan which indicates respiratory activity.

Figure 2 shows a schematic diagram of the use of magnetic beads in the method of the invention.

Figure 3 shows E. coli 0157:H7 cultures grown in CA-YE medium (a) to logarithmic phase (24hr) and (b) stationary phase (48hr). Cultures were diluted in PBS and enumerated by plate counts on different agar types.

Figure 4 shows agar plate counts and direct microscopic counts of 48-h CA-YE cultures of E. coli 0157:H7 which were centrifuged and (a) resuspended in M9 medium without a carbon source, and (b) incubated at RT for 48 h. Fill patterns and error bars are the same as for figure 3.

Figure 5 shows enumeration of 48-h CA-YE cultures of E. coli 0157:H7 which were centrifuged and (a) resuspended in reverse osmosis water, and (b) incubated

at RT for 48 h. Fill patterns and error bars are the same as for figure 3.

Figure 6 shows a regression of CTC-positive E. coli 0157:H7 cells counterstained with Fab, against R2A plate counts. Δ CA-YE logarithmic phase (24h) cultures; □ CA-YE stationary phase (48hr) cultures; o M9 suspensions before incubation; ◇ M9 suspensions after incubation for 48 h at RT. ---- regression line; ····· 99% confidence interval. R=0.997.

Figure 7 shows a Schematic of Presumptive Test for Evaluation of VTEC Contamination

Figure 8 shows a photomicrograph of cells of E. coli 0157:H7 reacted with rabbit anti-O157 primary antiserum and with goat anti-rabbit fluorescein conjugate after incubation with CTC*.

Disclosure of the Invention

It is thus an object of the present invention to provide a rapid method for the detection, identification and enumeration of respiring microorganisms.

It is a further object to provide a rapid method for testing potable water sources and water storage areas for microbial contaminants.

Additional objects of the invention will become apparent to one of skill in the art from the disclosure of the invention, below.

The present invention provides a rapid method for the detection, identification and enumeration of respiring bacteria and other microorganisms, including VTEC.

In a preferred embodiment the method comprises the steps of

a) passing a microbial sample through a collecting device to capture the cells;

b) adding to the collecting device a fluorochrome dye specific for the detection of respiring microorganisms and allowing the dye to incubate;

c) treating the collecting device with a reactive fluorescent antibody which reacts with target cells of interest present in said microbial sample;

d) mounting the collecting device for examination by fluorescence microscopy in which a suitable light filter system is used to excite the fluorochrome dye and fluorescent antibody to fluoresce; and

e) quantifying the respiring microbial cells.

Preferably the fluorochrome dye specific for the detection of respiring bacteria is taken up by respiring microorganisms and reduced to insoluble formazan crystals by the cytochrome system of said microbes. More preferably the fluorochrome dye specific for the detection of respiring cells is a tetrazolium compound. Most preferred is tetrazolium compound which is 5-Cyano-2,3-Ditolyl Tetrazolium Chloride (CTC).

Additionally the invention provides a rapid method for the detection, identification and enumeration of respiring target microorganisms comprising the steps of

a) filtering a sample through a membrane filter to capture cells;

b) transferring the membrane filter to a pad comprising a fluorochrome dye specific for the detection of respiring microorganisms;

c) incubating said microbial sample with said fluorochrome dye;

d) treating the surface of the membrane filter with a reactive fluorochrome labeled antibody which reacts with a specific microbial species or strain of interest present in said sample;

e) mounting said membrane filter for examination by fluorescence microscopy in which a suitable light filter

system is used to excite the fluorochrome dye and fluorochrome labeled antibody to fluoresce; and

f) quantifying said respiring target microbial cells.

Description of the Invention

SECTION I

Rapid methods which indicate physiological activity in bacteria include 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a fluorescent analogue of iodonitro tetrazolium chloride (INT) which indicates respiratory activity (Roderiguez, G.G., D. Phipps, K. Ishiguro & H.F. Ridgway. 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Appl. Environ. Microbiol. 58:1801-1808). Rhodamine 123 is another fluorochrome which indicates proton motive force (Kaprelyants, A.S. & D.B. Kell. 1992. Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. J. Appl. Bacteriol. 72:410-422).

2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium Chloride (INT) has been used in ecological and environmental studies to indicate respiratory activity in bacteria (Zimmerman, R., R.M. Iturriaga, and J. Becker-Birck. 1978 Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Applied and Environmental Microbiology 36:926-935.) The INT acts as an artificial electron acceptor and is converted to insoluble red crystals of INT-formazan within the cells of metabolically active bacteria. Another compound, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) has been developed (Stellmach and Severin, German Patent DE 3418852 A1) which is similarly converted to CTC-formazan crystals which are fluorescent when examined by epifluorescent microscopy using appropriate excitation,

barrier and emission filters. This method was used to assess the effects of disinfection of bacteria in biofilms (Yu, F.P., and G.A. McFeters. 1994 Rapid *in situ* assessment of physiological activities in bacterial biofilms using fluorescent probes. Journal of Microbiological Methods [in press]), and also adapted for use with bacteria collected in polycarbonate membrane filters.

In another approach, the direct viable count (DVC) method, cells are incubated for a few hours with appropriate nutrients and a quinoline antibiotic (usually nalidixic acid) which prevents division and hence causes viable (nutrient responsive) cells to elongate (Kogure, K., U. Simidu & N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25:415-420).

Respiring cells may also be enumerated by microcolony formation, where cells are immobilized on a polycarbonate membrane and incubated for a few hours on an appropriate medium which facilitates the development of microcolonies by nutrient responsive cells (Powell, E.O. 1956. A rapid method for determining the proportion of viable bacteria in a culture. J. Gen. Microbiol. 14:153-159; Postgate, J.R., J.E. Crumpton & J.R. Hunter. 1961. The measurement of bacterial viabilities by slide culture. J. Gen. Microbiol. 24:15-24).

CTC and rhodamine 123 were used for physiological assessment of biofilms following disinfection. (Yu, F.P., B. Pyle & G. McFeters. 1993. A direct viable count method for the enumeration of attached bacteria and assessment of biofilm disinfection, Journal of Microbiological Methods 17:167-180.) Rhodamine 123 and CTC have also been used in conjunction with flow cytometry (Diaper, J.P., K. Tither & C. Edwards. 1992. Rapid assessment of bacterial viability by flow cytometry. Appl. Microbiol. Biotechnol. 38:268-272;

Kaprelyants, A.S. & D.B. Kell. 1992. Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. J. Appl. Bacteriol. 72:410-422; Kaprelyants, A.S. & D.B. Kell. 1993. The use of 5-cyano-2,3-ditolyl tetrazolium chloride and flow cytometry for the visualization of respiratory activity in individual cells of *Micrococus luteus*. J. Microbiol. Meth. 17:115-122), and CTC has been used to quantify planktonic and sessile bacteria in drinking water.

All of these methods are non-specific, i.e. although they may be used with pure cultures of specific organisms, they will not differentiate between bacterial species when applied to mixed populations.

On the other hand, methods for detecting specific bacteria, e.g. fluorescent antibodies or oligonucleotide probes, have not been developed to indicate physiological activity or viability of bacteria.

Microcolony formation has previously been combined with immunofluorescence for the detection of viable *Listeria* (Sheridan, J.J., I. Walls, J. McLaughlin, D. McDowell & R. Welch. 1991. Use of a microcolony technique combined with an indirect immunofluorescence test for the rapid detection of *Listeria* in raw meat. Lett. Appl. Microbiol. 13:140-144) and *Salmonella* (Roderigues, U.M. & R.G. Kroll. 1990. Rapid detection of salmonellas in raw meats using a fluorescent antibody-microcolony technique. J. Appl. Bacteriol. 68:213-223). The direct viable count method has also been successfully combined with immunofluorescence for the detection of viable *Vibrio cholerae* (Brayton, P.R. & R.R. Colwell. 1987. Fluorescent antibody staining method for enumeration of viable environmental *Vibrio cholerae* O1. J. Microbiol. Meth. 6:309-314), and *Escherichia coli* and *Salmonella enteritidis* (Roszak, D.B. & R.R. Colwell. 1987. Metabolic activity of bacterial cells enumerated

by direct viable count. Appl. Environ. Microbiol. 53:2889-2983).

While the polymerase chain reaction has been widely employed for detection of natural bacterial populations in water (e.g. Bej, E.K., J.L. DiCesare, L. Haff, & R.M. Atlas. 1991. Detection of *Escherichia coli* and *Shigella* spp. in water by using a polymerase chain reaction and gene probes for uid. Appl. Environ. Microbiol. 57:1013-1017), the procedure is tedious and lengthy, requiring specialized thermal cycling equipment. Furthermore, no PCR method has been proposed for the reliable detection, enumeration and examination of individual viable cells. Oligonucleotide probes have been developed for a wide range of bacteria (Ward, D.M., M.M. Bateson, R. Weller & A.L. Ruff-Roberts. 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. In *Advances in Microbial Ecology*, Vol. 12, K.C. Marshall (ed.), pp. 219-286. New York, Plenum Press), and fluorescent 16s rRNA oligonucleotide probes have been investigated using an *E. coli* 16s rRNA sequence and two bacterial sequences in conjunction with DAPI for total direct counts in water samples (Hicks, R.E., R.I. Amann & D.A. Stahl. 1992. Dual staining of natural bacterioplankton with 4',6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. 58:2158-2163). Similarly, single bacterial cells have been identified using digoxigenin-labeled rRNA probes (Zarda, B., R. Amann, G. Wallner & K.-H. Schleifer. 1991. Identification of single bacterial cells using digoxigenin-labelled, rRNA-targeted oligonucleotides. J. Gen. Microbiol. 137:2823-2830), and fluorescently labeled oligonucleotide probes have been used for detection of microorganisms in soil (Hahn, D., R.I. Amann, W. Ludwig, A.D.L. Akkermans & K.-H. Schleifer. 1992 Detection of micro-organisms in soil after *in situ* hybridization with

rRNA-targeted, fluorescently labelled oligonucleotides. J. Gen. Microbiol. 138:879-887). Direct microscopic examination was employed following staining with DAPI, gel transfer, and hybridization with rhodamine-labeled probes. Although these techniques are rapid and specific, they are of little value in establishing respiration of bacteria and viability.

The present method involves the treatment and incubation of cells concentrated by a collecting device, such as a filter membrane, followed by microscopic examination to determine viability, respiration, quantification and identity of the microorganism. Physiological assessment is based on the reaction of fluorogenic substrates which permit the evaluation of the respiratory activity of the microorganism. The identification is performed with labeled antibodies or oligonucleotide probes.

Research on microscopic methods for the detection of bacteria in spacecraft water systems has led us to develop a method in which the CTC incubation is combined with a fluorescent antibody test (Figure 1). The method has been performed successfully with, for example, *E. coli* O157:H7 and *Salmonella typhimurium*. The method can be performed with any microorganism which can take up and metabolize CTC by respiratory cytochrome activity.

Briefly, cells in a liquid sample are collected on the surface of a 0.2 μm porosity polycarbonate filter membrane. The membrane is incubated at room temperature on a pad which is saturated with a buffer solution containing CTC. After 1-3 hours incubation, the membrane is fixed with formalin and heated. A drop of primary antibody which is specific for the target organism is applied and incubated for 20-30 min, followed by rinsing with buffer to remove unbound antibody. A drop of secondary fluorescent antibody conjugate which is specific for the primary antibody is applied and

incubated for 20-30 min. The membrane is rinsed and mounted in buffered glycerol for examination by epifluorescence microscopy. Using appropriate excitation/barrier/emission light filters, cells which have accumulated CTC-formazan crystals contain yellow/red spots, and those which have reacted with the fluorescein-conjugated antibody appear green (Figure 1).

The following is an example of the method according to the present invention.

EXAMPLE 1

A liquid sample containing bacteria is filtered through a membrane (black polycarbonate) to capture bacteria from the sample.

The membrane is transferred to a pad which is saturated with a medium containing a fluorochrome (5-Cyano-2,3-Ditolyl tetrazolium Chloride (CTC)) which indicates the viability of a cell when the fluorochrome is taken up and metabolized.

After incubation with a fluorochrome, the surface of the membrane filter is treated with a particular antibody which will react with the target bacteria. After reaction with the antibody, the membrane filter is rinsed to remove unbound antibody. The reactive antibody and fluorescent CTC formazan are then visualized.

In a preferred embodiment a gel blocking step may be used to suppress background hybridization in the fluorescent antibody labelling step of the method of the invention. To perform this step, one drop of hydrolyzed gelatin solution is gently spread over the membrane filter surface or collection device surface. The membrane is brought to near dryness. Gelatin is prepared by autoclaving a 2% gelatin solution at pH 10 followed by adjustment to Ph 7.2 and freezing in 1 ml aliquots. Prior to use, the hydrolyzed gelatin is diluted with distilled water and filter sterilized. (Cochran-Stafira,

D.L. & M. Starzyk. 1989. Membrane filter fluorescent antibody technique for the detection and enumeration of the genus *Thermus* in water. Microbios 60:159-165.) One drop of filter sterilized FITC-conjugated antibody, appropriately diluted with phosphate buffered saline, is added to the gel coated membrane. At the end of incubation the membrane filter is mounted for bacterial identification and quantification.

The membrane filter is examined by fluorescence microscopy in which a suitable light filter system is used to excite the viability and antibody-conjugated fluorochrome to fluorescence.

The bacteria may then be quantified by taking into account the original volume of the liquid filtered, the filtration area and the microscopic field area including the number of fields examined.

EXAMPLE 2

Bacterial Strains and Growth Conditions A culture of Escherichia coli 932, serotype O157:H7, was provided by the U.S. Environmental Protection Agency, Cincinnati, OH. The culture was streaked on MacConkey Sorbitol agar (MSA) and Tryptone Lactose Yeast Extract (TLY) agar (42) to determine purity, harvested from TLY agar into 20% glycerol-2% peptone and frozen (-70°C) as stocks. Identity was confirmed by API 20E test strips (bio Mérieux, Hazelwood, MO). Unless specified otherwise, all media and solutions were prepared with reagent grade water (Milli-Q UV Plus, Millipore Corp.) and sterilized by autoclaving at 121°C for 20 min.

Frozen stock cultures were inoculated into 100 ml volumes of medium containing 0.3% casamino acids and 0.03% yeast extract (CA-YE) in side-arm flasks, at ca. 10^5 CFU/ml starting inoculum and incubated at room temperature (RT) with shaking at 100 rpm for 24-25 h to obtain cells in mid to late logarithmic phase with

continued incubation to stationary phase at 48-50 h. Optical density readings (Klett-Summerson Photometric Colorimeter) were made periodically throughout the incubation to confirm the phase of growth.

For starvation experiments, 5 ml of 48 h CA-YE culture was centrifuged with refrigeration at ca. 3000 x g for 15 min and resuspended in 5 ml of sterile distilled water. These suspensions were diluted to obtain ca. 105 target cells/ml in 100 ml of either sterile M9 medium without nutrients (37) or Millipore reverse osmosis (RO) water. Fresh RO water, which normally contains 102-103 CFU/ml of heterotrophic bacteria, was incubated at RT on a shaker at 100 rpm for 5 days to develop a stable bacterial population. Stabilized RO water usually contained ca. 105 cells/ml, and it was desirable to inoculate with about an equal number of E. coli O157 cells. Thus, the target number of 105 E. coli O157 cells/ml was adopted. The M9 or RO cell suspensions were incubated at RT on the shaker for an additional 48 h. The sterile M9 medium was used to determine the effects of starvation on the CTC and FA reactions, and the RO water to determine the effects of starvation and competition by indigenous heterotrophic microorganisms on FA specificity.

Cell Suspension Preparation

An aliquot of CA-YE culture was diluted to 10-1 or 10-2 in 0.22 μm filtered (Millipore type GS) autoclaved phosphate buffered saline (PBS, pH 7.5) and vortexed at full speed for 1 min (Fisher Vortex Genie 2). A sample of this suspension was diluted in PBS (usually a further 10-2 or 10-3 dilution) to obtain ca. 105 cells/ml. This suspension was decimally diluted in sterile distilled water blanks for enumeration by a modified drop plate method (35) on R2A agar (1), TLY agar, TLYD agar (TLY with deoxycholate) (42), MSA and MacConkey lactose agar

(MLA). Cell injury was estimated as the percentage difference between counts on the TLY and TLYD agar media. The same suspension was membrane filtered and examined by staining with DAPI, the CTC reduction assay, FA staining, and the combined CTC/FA procedures described below.

DAPI Staining

The cell suspension (usually 5 or 10 ml) was filtered through a black polycarbonate membrane (25 mm diam., 0.2 μm porosity, Nuclepore). The membrane was transferred to a 25 mm absorbent pad (Millipore) saturated with 0.6 ml sterile water and 0.1 ml 37% formaldehyde and incubated for at least 5 min at room temperature to fix the cells. The membrane was replaced on the filter apparatus, covered with 0.5 ml of a 10 μg/ml prefiltered solution 4,6- diamidino-2-phenylindole (DAPI; Sigma), and incubated for 5 min before vacuuming off the stain. This DAPI concentration and staining time are well within the ranges used by others (22). The membrane was placed in a small petri dish on a 47 mm absorbent pad (Millipore) and allowed to dry. Dried membranes were kept under refrigeration for up to 7 days before examination. Immediately before microscopic examination, the membrane was placed on a drop of carbonate buffered glycerol (pH 9; glycerol with 0.05M carbonate buffer) on a microscope slide, and another drop of glycerol placed on top followed by a coverslip which was firmly pressed down to flatten the membrane filter. Filters were examined under oil immersion epifluorescence using a Leitz Ortholux II microscope with Leitz Filter Block B2 (excitation 350-410 nm, dichroic mirror 455 nm, suppression filter 470 nm).

Membrane Filter CTC Reduction Assay

This was performed as described elsewhere (8). Essentially, the cell suspension was filtered through a black polycarbonate membrane, and the membrane filter was transferred to an absorbent pad saturated with 0.85% physiological saline (PS) containing 5 mM CTC (5-cyano-2,3-ditolyl-tetrazolium chloride; Polysciences). Following incubation at room temperature for 1.5 h, the membrane filter was lifted and 0.1 ml formaldehyde (37%) dispensed onto the pad, the membrane filter replaced and incubated for at least 5 min. The membrane filter was stained with DAPI on a filter apparatus, dried on an absorbent pad and mounted in buffered glycerol under a coverslip. The mounted filter was examined by epifluorescence microscopy (Leitz Ortholux II) using light filters for DAPI (Leitz Filter Block B2) or CTC (Leitz Filter Block N2.1; excitation 515-560 nm, dichroic mirror 580 nm, suppression 580 nm). Both the DAPI stain and CTC formazan crystals could be observed together by using Filter Block H (excitation 420-490 nm, dichroic mirror 510 nm, suppression 520 nm).

Fluorescent Antibody Staining

The cell suspensions were filtered as described above, transferred to a pad saturated with PS and formaldehyde, and incubated for at least 5 min. The membrane was transferred to a small petri dish which was placed on a slide warmer (Fisher) at ca. 65°C for 10 min, then allowed to cool. For E. coli O157:H7, a drop (50 µl) of fluorescein isothiocyanate (FITC) conjugated O157 antibody (Kirkegaard & Perry Laboratories) diluted 1:100 in PBS was placed on the membrane filter, and spread with the top end of a 200 µl dispenser tip. The petri dish lid which contained the saturated pad was replaced to form a humidity chamber for the 20 min incubation. The membrane filter was gently removed from the dish,

transferred to a filtration apparatus and washed with three 0.5 ml volumes of PBS. The filter was either dried and stored as stated above or mounted in buffered glycerol under a coverslip for microscopic examination. Epifluorescence microscopy was performed as above using Leitz filter block H. FA method controls were prepared by substituting PBS for the FITC-conjugated FA solution, followed by staining with DAPI to determine if the FA procedure resulted in any changes in total cell numbers.

Combined CTC Reduction and Fluorescent Antibody

Cell suspensions were filtered and incubated with CTC for 1.5 h as described above, then transferred to a pad saturated with PS and formaldehyde. After 5 min incubation with formaldehyde, the filter was heat fixed, reacted with FA for 20 min, rinsed and mounted for microscopic examination. Leitz filter block H was used to visualize both CTC-formazan crystals and FA-positive cells, and filter block N2.1 was utilized to confirm and enumerate the CTC-positive cells. The direct FA O157 procedure results were compared with the number of FA positive cells for the combined CTC-FA technique to determine if the CTC incubation affected the FA results.

Table 1 provides a third example of the method of the present invention.

TABLE 1

Procedure for detection of cells which reduce CTC and react to a specific fluorescent antibody, using polycarbonate membrane.

Pass sample or diluted culture through a 0.2μm porosity black polycarbonate membrane to obtain 10-100 cells in a microscope field.

↓

Transfer membrane to a pad saturated with medium containing CTC and incubate at room temperature

↓

Fix by lifting the membrane and adding 0.1 ml 37% formalin to the pad, replacing the membrane and incubating at least 5 min

↓

Place membrane in glass petri dish, heat for 10 min at ca. 65°C

↓

Place a drop of gelatin solution on membrane, spread, heat at ca. 55°C until almost dry; allow to dry and cool at room temperature

↓

Place a drop of diluted antiserum on the membrane, spread, and incubate in an enclosed petri dish for 30 min at room temperature

↓

Transfer membrane to filter funnel apparatus, wash with two 0.5 ml volumes of PBS

↓

Replace membrane in petri dish, place a drop of fluorescent conjugated anti-antiserum on the membrane, spread and incubate in closed dish for 30 min at room temperature

↓

Transfer membrane to filter funnel apparatus, wash with three 0.5 ml volumes of PBS

↓

Mount filter in buffered glycerol, add coverslip and examine with epifluorescence using 100% objective under oil immersion; use light filters appropriate for the fluorochrome which was conjugated to the anti-antiserum.

Viable E. coli O157:H7 cells were mounted on black polycarbonate filters and stained with DAPI following incubation with CTC appeared blue-green with bright red/orange spots of CTC-formazan when examined by epifluorescence microscopy using Leitz filter block H. Similar results were observed with CTC/FA stained cells, although the cells appeared larger because of the FA attached to extracellular antigenic material.

Logarithmic and Stationary Phase Cultures

Plate counts on R2A and TLY agar increased by less than one log between the 24 and 48 h incubation times (Fig. 3). Optical density readings showed that the CA-YE culture was in the mid- to late-logarithmic phase of growth at around 24 h incubation and had reached stationary phase before 48 h. The final cell concentrations in this relatively low nutrient medium (0.3% casamino acids, 0.03% yeast extract) were ca. 109 CFU/ml. Growth on TLYD agar, MSA and MLA was severely restricted for the 24 h cultures but not for the 48 h samples (Fig. 3). There was over 90% injury in the actively growing 24 h cultures (Table 2), while less than 20% injury was detected for the stationary phase 48 h cultures. This observation is consistent with chemostat data which show that faster growing bacteria are more sensitive to antimicrobial agents (24). The MacConkey media also detected almost 100-fold fewer E. coli O157 cells in the 24 h cultures than the non-selective R2A and TLY media (Fig. 3). The MacConkey media, like TLYD agar, contain the bile salt surfactant deoxycholate which is well known for its inhibitory effect on target bacteria (25). It is interesting to note that in this study, the actively growing cultures were most sensitive to the selective effects of these media.

Suspensions of CA-YE cultures diluted in PBS at 24 and 48 h of incubation stained only with DAPI, and those

incubated with CTC before DAPI staining, increased in total cell numbers by ca. one-half log from 24 to 48 h (Fig. 5). More than 90% of cells in the logarithmic phase (24-h) sample were CTC-positive, and 70-80% of stationary phase (48-h) cells reduced CTC (Table 2).

TABLE 2: Percentages of injured and CTC-reducing cells. Suspensions of E.coli O157:H7 cells were prepared with PBS after incubation in CA-YE medium (mid-logarithmic growth phase, 24-hour culture, and stationary phase, 48-hourculture). Suspensions of cells from stationary phase cultures were also examined before and after incubation in M9 medium or reverse osmosis (RO) water for 48 hours at room temperature. Results are shown as the mean+standard error (n=4).

	CA-YE Cultures		Before Incubation		After Incubation	
	24-hour	48-hour	M9	RO	M9	RO
% Injury[1]	91.4+ 6.1	16.1+ 8.5	4.9+ 5.2	6.0+5.6	24.9+12.0	17.2+13.1
% CTC+/DAPI+[2]	90.8+ 2.3	79.9+13.0	85.5+ 8.5	82.1+7.4	79.8+ 5.2	55.5+ 6.2
% CTC+/FA+[3]	91.0+ 1.5	69.7+11.4	71.3+10.5	80.3+ 4.0	78.0+ 2.5	76.2+7.4

[1] ((TLY CFU - TLYD CFU) / (TLY CFU) x100;

[2] (CTC-Positive, DAPI-stained cells/DAPI-stained)x100;

[3] (CTC-Positive, FA-stained cells/FA-stained)x100.

TABLE 3: Ratios of E. coli O157:H7 cell counts determined by the CTC-DAPI,FA and CTC-FA[1] procedures compared to DAPI-stained controls. Cultureswere prepared as described in Table 1. Results are shown as the mean+standard error (n=4).

	CA-YE Cultures		Before Incubation		After Incubation	
	24-hour	48-hour	M9	RO	M9	RO
CTC-inc,DAPI[2]	1.35+0.11	1.21+0.23	1.28+0.09	1.15+0.15	1.00+0.06	0.82+0.17
CTC+,DAPI[3]	1.23+0.10	0.88+0.09	1.10+0.02	0.94+0.12	0.79+0.05	0.48+0.12
FA Control[4]	1.28+0.17	0.65+0.18	1.39+0.49	0.82+0.13	0.91+0.05	0.48+0.16
CTC-inc,FA+[5]	1.17+0.10	0.95+0.15	1.53+0.40	0.93+0.24	0.97+0.10	0.41+0.17
CTC+,FA+[6]	1.07+0.10	0.61+0.12	1.06+0.20	0.79+0.20	0.84+0.09	0.45+0.27

[1] DAPI-stained control (no CTC or FA);

[2] cells stained with DAPI after CTCincubation;

[3] CTC-positive, DAPI-stained;

[4] FA control (no CTC or DAPI);

[5] FA-stained cells after CTC incubation;

[6] CTC-positive, FA-stained cells.

DAPI staining of cells after CTC incubation yielded slightly higher counts compared to simple DAPI staining for both 24-h and 48-h CA-YE cultures (Fig. 3, Table 3). The numbers of CTC-reducing cells enumerated by the CTC-FA technique were almost identical to those determined by the CTC- DAPI method for the growing cultures and about 40% fewer were detected in the stationary phase (Table 3). Performing the FA procedure following CTC incubation did not appreciably affect the numbers of CTC-positive cells, excepting the stationary phase cultures.

M9 Medium and Reverse Osmosis Water Suspension

When stationary phase cells were centrifuged, resuspended in M9 medium and incubated on a shaker for 48 h at room temperature to facilitate starvation, there was a slight increase in viable plate counts (Fig. 4). Little injury (ca. 5%) was detected in the M9 or RO water suspensions before incubation, and about 20-25% of these populations were injured after incubation (Table 3). The MSA counts of sorbitol-negative colonies in the RO water suspension samples confirmed that the R2A and TLY agar plate counts were comprised almost totally of the introduced E. coli O157 cells which outnumbered the

natural bacteria population in RO water, according to plate count data (Fig. 5).

Total DAPI cell counts in M9 medium and, more obviously, in RO water after 48 h incubation increased somewhat from those at the time of inoculation (Figs. 4 and 5). Interestingly, the plate counts on media including non- selective R2A and TLY did not indicate this increase (Fig. 5), even though the plates were incubated at room temperature for 5-7 days. The apparent increases in cell numbers in M9 medium and RO water might be attributed to the use of endogenous reserves in the cultured E. coli O157 cells, because the M9 medium was not supplemented with a carbon source. It is also possible that the increases observed with DAPI resulted from growth of RO water subpopulations which were not detected on R2A or TLY agar. In RO water, the larger increase in DAPI counts following incubation (Fig. 5) compared to the slight increase in FA counts suggested that heterotrophic bacteria which were natural contaminants in the RO water, rather than the introduced E. coli O157, grew during incubation. Almost 50% of the O157 cells were CTC-negative after incubation in RO water (Table 3). The DAPI-stained cells included the natural population of RO water organisms other than the introduced E. coli. Immediately after the M9 cell suspension was prepared, the FA control counts and CTC-incubated, FA-stained counts were up to 1.5-fold greater than the DAPI control counts (Table 3). This suggested that DAPI staining did not detect all the E. coli O157 cells when they had been centrifuged and resuspended in M9 medium. After incubation in M9 medium, the DAPI control counts were only slightly greater than the FA counts (Table 3). The inventors concluded that the FA counts are at least equivalent to, if not greater, than DAPI counts on similar populations.

During incubation in RO water, the increased numbers of DAPI-stained cells indicated that t he indigenous bacterial population multiplied (Fig. 5). The proportion of cells staining with either DAPI following CTC incubation, or those stained by FA methods decreased (Table 3). In addition, less than half of the RO-incubated cells detected by DAPI- or FA-staining after CTC incubation indicated respiratory activity by reducing CTC.

Regression of respiring E. coli O157:H7 cells enumerated by the CTC/FA technique compared with R2A agar plate counts showed that the methods gave comparable results over a wide range of cell concentrations (Fig. 6). Most values fell within the 99% confidence interval, and the regression coefficient was 0.997. This analysis included logarithmic and stationary phase cells diluted in PBS, stationary phase cells which had been washed and resuspended in M9 medium, as well as the same populations after incubation for 48 h in M9. Cell injury, which was detected by enumeration on TLY and TLYD agar plates, varied significantly between these populations (Table 1), while the CTC counts were generally in agreement with R2A agar plate counts (Fig. 6). These results suggest that cell injury did not significantly affect CTC reduction.

The FA technique is an established method for detecting and identifying species which have been cultured and for which antibody preparations are available. Danielsson et al (10,11) adapted the FA procedure for use with membrane filters, and others have successfully applied this approach in combination with the direct viable count (12) and microcolony method (37) to enumerate viable pathogenic bacteria. The FA method has also been used in combination with INT staining to detect specific respiring bacteria (2).

Incubation with CTC to detect respiratory activity was originally proposed for Erlich's ascites tumor cells

(45), and has since been used to enumerate respiring bacteria in water (8,38,40). Respiratory activity in populations of enteric bacteria in a polar marine environment (44), and native bacteria in soil (48), has also been examined. The viability of Pseudomonas fluorescens (19) and coccoid Campylobacter jejuni (5) have likewise been determined using this technique. Flow cytometry has been used to enumerate Micrococcus luteus following CTC incubation (19). The technique has been applied to the examination of bacterial biofilms in situ on solid surfaces (49,50). CTC is becoming more widely used in microbial ecology and physiology investigations, and we are learning more about its characteristics as an indicator of bacterial respiratory activity (6,38,40,43). The procedure described incorporates the advantages of membrane filtration for sample concentration, incubation of the membrane on an absorbent pad saturated with CTC medium for assessment of respiratory activity, and reaction with a FA directly on the membrane for detection of a specific organism which was, in this case, E. coli O157:H7. A similar method, using an indirect FA reaction, has been applied successfully to cultures of Salmonella typhimurium and Klebsiella pneumoniae. The time taken from sampling to completion of epifluorescent microscopy ranges from 3-4 hours, which is significantly more rapid than many other methods. Discrimination of the specific respiring cells is relatively straightforward when the FA is conjugated with a fluorochrome such as fluorescein isothiocyanate which gives contrasting fluorescence with the appropriate epifluorescence microscopy filters. Results of the CTC/FA technique compared favorably with plate counts using non-selective R2A medium.

An advantage of the present method is that it is relatively rapid and minimizes actual labor input to about 1 to 2 hours with a total assay time of 3 to 6

hours. Several samples can be processed at the same time. The procedure is amenable to automated examination using video image analysis technology. The method provides for the rapid detection, identification and enumeration of respiring bacteria.

In the alternative, incubation of CTC can be performed by spraying or liquid application onto the surface of a membrane. Similarly antibodies or probes can be applied by other means known to those of skill in the relevant art. In an alternative embodiment, the collecting device to capture the bacteria from a contaminated sample comprises a centrifuge for performing sedimentation field flow fractionation. The method of sedimentation field-flow fractionation is known to those of skill in the art (see Sharma et al., "Physical Characterization and Quantification of Bacteria by Sedimentation Field-Flow Fractionation", Applied and Experimental Microbiology, Vol. 59, No. 6, June 1993, pp 1864-1875, incorporated herein by reference in its entirety)."

EXAMPLE 3

Incubation for CTC reduction, cell elongation or microcolony formation was performed, followed by fluorescent antibody (FAb) reaction or nucleic acid hybridization directly on polycarbonate filter membranes. Both fluorescent antibody techniques (Cochran-Stafira, D.L. & M.J. Starzyk. Membrane-filter fluorescent antibody technique for the detection and enumeration of the genus *Thermus* in water. Microbios 60:159-165; Desmonts, C., J. Minet, R. Colwell & M. Cormier. 1990. Fluorescent-antibody method useful for detecting viable but nonculturable *Salmonella* spp. in chlorinated wastewater. Appl. Environ. Microbiol. 56:1448-1452) and oligonucleotide probe methods (Heidelberg, J.F., K.R. O'Niel, D. Jacobs & R.R. Colwell. 1993. Enumeration of

Vibrio vulnificus on membrane filters with a fluorescently labeled oligonucleotide probe specific for kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. 59:3464-3476) have been performed directly on filter membranes.

A protocol we have developed for the CTC/Fab approach is shown in Table 1. A photomicrograph (Fig. 1) indicates the visual appearance of a typical sample prepared by the protocol outlined in Table 1.

The viability/nucleotide probe method is based on initial incubation for microcolony formation. Cells which respond by growing into microcolonies have increased rRNA content and because there are several cells they are more easily detected.

Automation and electronic data capture and processing are desirable in the context of water quality monitoring in the spacecraft environment. The inventors have previously developed methods for rapid enumeration of viable bacteria by image analysis (Singh, A., B.H. Pyle & G.A. McFeters. 1989. Rapid enumeration of bacteria by image analysis epifluorescence microscopy. J. Microbiol. Meth. 10:91-102; Singh, A. F.P. Yu & G.A. McFeters. 1990. Rapid detection of chlorine-induced injury by the direct viable count method using image analysis. Appl. Environ. Microbiol. 56:389-394). These procedures were based on the direct viable count technique (Kogure, K., U. Simidu & N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25:415-420). The combined methods of the present invention are also amenable to quantification by image analysis.

In an alternative embodiment, the invention provides for a method for the detection, identification and enumeration of a respiring target bacterium comprising the steps of

a) mixing immunomagnetic beads comprising an antibody which specifically binds to a target bacteria with a liquid sample comprising said target bacteria;

b) allowing said liquid sample to interact with the beads for up to an hour;

c) placing the sample in a magnetic separator which causes the magnetic beads to which target bacteria have attached to separate from the liquid sample;

d) aspirating the liquid from the liquid sample, leaving the beads with bacteria attached;

e) washing the beads with a solution which removes loosely bound bacteria and other particles from the liquid sample;

f) mixing beads with bacteria attached with a fluorochrome dye specific for the detection of respiring bacteria;

g) treating bacteria on the beads with a fluorescent stain or a specific fluorescent conjugated antibody;

h) mounting said sample for examination by epifluorescent microscopy, in which a suitable light filter system is used to excite the fluorochrome dye and fluorochrome labeled antibody to fluoresce; and

i) quantifying said respiring target bacteria.

Again it is preferred that the fluorochrome dye specific for the detection of respiring bacteria is taken up by respiring bacteria and reduced to insoluble formazan crystals by the cytochrome system of the bacteria, such as 5-Cyano-2,3-Ditolyl Tetrazolium Chloride (CTC).

Materials are commercially available which can facilitate concentration and separation of target bacteria from food homogenates. The technique is known as immunomagnetic separation (IMS).

Immunological separation of bacteria from food samples using specific antibodies coated on a variety of surfaces has led to the use of magnetizable particles

(Blackburn, C. de W. 1993 Rapid and alternative methods for the detection of salmonellas in foods. Journal of Applied Bacteriology 75:199-214). IMS techniques utilize small particles or beads coated with antibodies against surface antigens of specific bacteria (Olsvik, O., T. Popvic, E. Skjerve, K.S. Cudjoe, E. Hornes, J. Ugelstad, and M. Uhlen. 1994 Magnetic separation techniques in diagnostic microbiology. Clinical Microbiology Reviews 7:43-54). The super-paramagnetic beads become magnetic when in a magnetic field but become nonmagnetic as soon as the field is removed. Thus, the particles remain in suspension when they are not in a magnetic field, and can be readily concentrated by applying a magnetic field. Isolation of specific bacteria bound to beads has usually been accomplished by cultivation of captured cells in broth or on solid media. Bacteria bound to magnetic beads remain viable when provided with adequate nutrients.

IMS has been used to isolate a variety of bacteria including *E. coli* K88 (Lund, A., A.L. Hellemann, and F. Vartdal. 1988 Rapid isolation of K88$^+$ *Escherichia coli* by using immunomagnetic particles. Journal of Clinical Microbiology 26:2572-2575), *Salmonella* spp. (Blackburn and Patel, 1989; Skjerve and Olsvik, 1991), *Listeria monocytogenes* (Skjerbe et al., 1990), and *Vibrio parahaemolyticus* serotype K (Tomoyasu, T. 1992 Development of the immunomagnetic enrichment method selective for *Vibrio parahaemolyticus* serotype K and its application to food poisoning study. Applied and Environmental Microbiology 58:2679-2682). *Shigella dysenteriae* and *S. flexneri* in feces were detected by immunomagnetic assay with monoclonal antibodies (Islam, D., S. Tzipori, M. Islam, and A.A. Lindberg. 1993 Rapid detection of *Shigella dysenteriae* and *Shigella flexneri* in feces by an immunomagnetic assay with monoclonal antibodies. European Journal of Clinical Microbiology

and Infectious Diseases 12:25-32). Okrend et al. (Okrend, A.J.G., B.E. Rose, and C.P. Lattuada. 1992 Isolation of *Escherichia coli* O157:H7 using O157 specific antibody coated magnetic beads. Journal of Food Protection 55:214-217) found that *E. coli* O157:H7 could be sensitively and specifically concentrated from ground beef by using magnetic beads coated with O157 antibody. The cells were subsequently cultivated in a nonselective growth medium. *E. coli* O157:H7 strains have also been extracted from enrichment broths (Fratamico, P.M., F.J. Schultz, and R.L. Buchanan. 1992 Rapid isolation of *Escherichia coli* O157:H7 from enrichment cultures of foods using an immunomagnetic separation method. Food Microbiology 9:105-113). The number of *E. coli* O157 recovered was related to the number of *E. coli* O157 in the sample. The sensitivity of recovery of *E. coli* O157:H7 was 10 CFU/ml in the enrichment medium. It was demonstrated that *E. coli* O157:H7 cells attached to the beads could be visualized by incubation with FITC labeled polyclonal antiserum against *E. coli* O157:H7 for 30 min followed by epifluorescent microscopic examination. Magnetic beads may be obtained commercially (e.g. Dynal, New York), and these may be supplied coated with the *E. coli* O157 antibody.

Essentially, *E. coli* O157:H7 bacteria in hamburger meat can be concentrated by immunomagnetic capture, as has been done with salmonellae (Vermunt, A.E.M., A.A.J.M. Franken, and R.R. Beumer. 1992 Isolation of salmonellas by immunomagnetic separation. Journal of Applied Bacteriology 72:112-118). After separation of the beads (with bacteria attached) from the meat suspension, the bead/cell particles are trapped on a filter membrane. The membrane is incubated for a few hours with a tetrazolium compound which, when taken up into respiring cells is reduced to a fluorescent formazan crystal. Reaction of the cells with a contrasting fluorescent

antibody permits the *E. coli* cells to be specifically labelled by the fluorescent dye. Subsequent direct microscopic observation and enumeration of the sample on the membrane filter permits discrimination of cells which were both metabolically active and of the specific O157:H7 antigenic type.

An important feature of the method of the invention is that it will discriminate between viable respiring bacteria which are possibly infectious and non-respiring cells which would have little likelihood of causing infections.

The proposed method has a number of advantages over other techniques, including:

The immunomagnetic capture step permits not only cell concentration, but also the selection of a specific antigenic cell type. Use of the *E. coli* O157 antigen concentrates bacteria that are likely to produce illness while eliminating other bacterial species from the bead concentrate.

The short term incubation with the respiratory fluorochrome provides rapid evaluation of the physiological status of the cells captured, indicating the potential for proliferation of the pathogen in a stored food product and its ability to infect consumers.

Reaction with a second fluorescently labelled antibody provides confirmation that the bacteria selected were indeed the target pathogen. For example, *E. coli* O157 antibodies different from the primary capture O157 antibody, e.g. from another manufacturer, confirm the presence of *E. coli* O157. Alternatively, a different antigen can be used for the confirmation, such as the H7 flagellar antigen or a fimbrial antigen. A similar approach can be taken when a pathogen other than *E. coli* O157 is the target organism.

Epifluorescence microscopic examination allows the enumeration of individual viable cells of *E. coli* O157,

in contrast to cultivation and enrichment techniques in which each colony may be formed by more than one cell.

The time required to complete the immunomagnetic concentration, viability incubation, fluorescent antibody reaction and microscopic examination is approximately 4-6 h. The procedure can be automated at several steps, reducing the time required to as little as three hours. These time requirements reflect a truly rapid method, allowing meat samples to be briefly withheld until results of the test become available.

For the simple, rapid detection of *E. coli* O157:H7 in ground beef, meat samples are added to a suitable extraction medium, e.g. sterile physiological saline containing 5 mg/ml (final concentration) of Protamine (Okrend, A.J.G., B.E. Rose, and C.P. Lattuada. 1992 Isolation of *Escherichia coli* O157:H7 using O157 specific antibody coated magnetic beads. Journal of Food Protection 55:214-217). Following brief (5-10 min) processing in a Stomacher, the homogenate is transferred to a tube and a suspension of anti-*E. coli* O157 0.28 μm diameter Dynabeads (Dynal, New York) added. The suspension is incubated with the beads for 10-60 min, then placed in a magnetic particle concentrator (Dynal) to immobilize the beads and captured cells.

After 1-5 min, the liquid meat/buffer suspension is removed by aspiration. The tube is taken off the magnetic separator and the beads resuspended in fresh buffer and washed. The CTC incubation can be carried out either directly with the bead/cell suspension or after collection of the bead/cell particles on a polycarbonate filter membrane. Filtration can prevent the loss of target cells during subsequent manipulations. This is followed by incubation with fluorescently-labelled *E. coli* O157 antibody, rinsing and mounting for epifluorescent microscopic examination and counting.

The number of fluorescent antibody positive bacterial cells which contain CTC-formazan crystals is counted as the number of viable *E. coli* O157 cells recovered. Non-viable cells (CTC-negative) are also counted to permit quantification of the total number of *E. coli* O157 in the meat sample.

Variations of the technique include the use of alternative, and possibly multiple, confirmatory fluorescent antibodies. Alternatives include antibodies for the *E. coli* H7 flagellar antigen, or a fimbrial antigen (Levine, M.M. 1987 *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. Journal of Infectious Diseases 155:377-39).

Different homogenization buffers can be used to obtain optimal immunomagnetic recovery of bacteria. Other than physiological saline containing protamine (Okrend, A.J.G., B.E. Rose, and C.P. Lattuada. 1992 Isolation of *Escherichia coli* O157:H7 using O157 specific antibody coated magnetic beads. Journal of Food Protection 55:214-217), plain physiological saline without Protamine may be used. Other possibilities include phosphate buffered saline (Doyle, M.P. and J.L. Schoeni. 1987 Isolation of *Escherichia coli* O157:H7 from retail fresh meat and poultry. Applied and Environmental Microbiology 53:2394-2396; and Lund, A., A.L. Hellemann, and F. Vartdal. 1988 Rapid isolation of K88$^+$ *Escherichia coli* by using immunomagnetic particles. Journal of Clinical Microbiology 26:2572-2575) with and without Protamine, and phosphate-buffered peptone water with 0.05% Tween 20 (Skjerve, E., and O. Olsvik. 1991 Immunomagnetic separation of Salmonella from foods. International Journal of Food Microbiology 14:11-18) or with 5 mg/ml protamine substituted for the Tween 20.

The attachment procedure may be varied. One minute vortexing and 10 min stationary incubation

followed by vortexing (Okrend, A.J.G., B.E. Rose, and C.P. Lattuada. 1992 Isolation of *Escherichia coli* O157:H7 using O157 specific antibody coated magnetic beads. Journal of Food Protection 55:214-217) may be used. Room temperature incubation with shaking for 10 min (Vermunt, A.E.M., A.A.J.M. Franken, and R.R. Beumer. 1992 Isolation of salmonellas by immunomagnetic separation. Journal of Applied Bacteriology 72:112-118) or with rotation for 15 or 60 min (Fratamico, P.M., F.J. Schultz, and R.L. Buchanan. 1992 Rapid isolation of *Escherichia coli* O157:H7 from enrichment cultures of foods using an immunomagnetic separation method. Food Microbiology 9:105-113) may also be used.

Direct CTC incubation of cells on beads in suspension or incubation following filtration can be used. Different membrane filters, e.g. Millipore or Nuclepore black polycarbonate membranes can be used in the present method for the filtration treatments.

CTC incubation media containing phosphate, e.g. R2A (Reasoner, D.J., and E.E. Geldreich. 1985 A new medium for the enumeration and subculture of bacteria from potable water. Applied and Environmental Microbiology 49:1-7) broth, can inhibit CTC reduction. Physiological saline can be used as a medium or a mixture of casamino acids (0.3%) and yeast extract (0.03%) (Singh, A., B. Pyle, and G. McFeters. 1989 Rapid enumeration of viable bacteria by image analysis. Journal of Microbiological Methods 10:91-101). Other more appropriate formulations may be obvious to one skilled in the art.

The CTC concentration may be varied over the range 1-5 mM. The preferred concentration for optimal reduction usually occurs in the range 2-5 mM.

The application of multiple confirmatory fluorescent antibodies, and direct vs. indirect fluorescent antibody techniques can be used. When multiple fluorescent antibodies are used, they are conjugated with contrasting

fluorescent compounds. The epifluorescent excitation/barrier/emission filter combinations may also be varied in the present method as is understood by those of skill in the art.

Incubation with CTC to detect respiratory activity is compatible with immunomagnetic capture and subsequent fluorescent antibody confirmation. The method does not involve a cultivation step, and there is a reasonable correlation with conventional culture methods. Optimization of the procedures should enable detection of very low numbers of target bacteria in ground beef.

While detection of less than 1 colony forming unit per gram of meat is expected, it has been found that levels of 10-6200 individual *E. coli* 0157:H7 per gram have been detected in beef samples associated with foodborne illness (Todd et al, 1988). Thus, a rapid, specific method which can detect 10 viable *E. coli* 0157 per gram sample would be useful in the identification of meats which were likely to cause food-borne disease.

The specificity of *E. coli* 0157:H7 antigens is debated. The advantage of the present method is that two antibodies can be used, one for the initial capture of the target bacteria on the immunomagnetic beads, and the other for confirmation after the incubation for respiratory activity. Thus, if a broad-spectrum antibody which may cross-react with some other species is used as the immunomagnetic bead antibody, this would help to optimize detection. A more specific fluorescent antibody can then be used for confirmation.

The proposed procedure, as outlined above, could be modified at several points to enhance its potential application. For example, as new specific antibodies become available, they could be used for the immunomagnetic separation step. If an antibody were available which would detect a wide range of verotoxigenic *E. coli*, including 0157:H7, this would

provide a more realizable assessment of the presence of enterohemorrhagic pathogens (Milley and Sekla, 1992).

The methods described above can also be modified for the detection of, for example, the pathogenic bacterium *Legionella pneumophila* which has been detected by immunofluorescence (Edelstein, P.H. 1993. Laboratory diagnosis of Legionnaires Disease: an update from 1984. In Legionella current Status and Merging Perspectives, Barbaree, J.M., Breiman, F.R., and Dufour, A.P. (Eds.), American Society for Microbiology, Washington, 7-11; Verissimo et al. 1991. Distribution of *Legionella* spp. in hydrothermal areas in continental Portugal and the Island of Sao Miguel, Azores. *Applied and Environmental Microbiology* 57:2921-2927).

The fluorescent tetrazolium compound CTC was originally used to detect respiratory activity in eukaryotic cells (Severin, Stellmach and Nachtigal. 1985. Fluorimetric assay of redox activity in cells. *Analytica Chimica Acta* 170:341-346), and similar respiratory systems exist in protozoan species which are also eukaryotes (Sleigh, M.A. 1991) The nature of protozoa. In Parasitic Protozoa, Second Edition, Volume 1, Kreier, J.P. and Baker, J.R. (Eds.), Academic Press, Inc., San Diego, P. 13). Immunofluorescent methods for detection and enumeration of the parasitic microbial protozoan species *Cryptosporidium* and *Giardia* (Rose et al. 1989. Evaluation of immunofluorescence techniques for detection of *Cryptosporidium* oocysts and *Giardia* cysts from environmental samples. *Applied and Environmental Microbiology* 55:3189-3196) can be combined with fluorescent indicators of cell activity such as CTC as described above for bacterial species.

Fluorescent probes, other than CTC, which assay cellular physiological activity can be used. These include Rhodamine 123, which provides an estimation of transmembrane potential, or fluorescein diacetate which

indicates esterase activity. These fluorochromes and others are discussed by McFeters et al (McFeters, G.A., Yu, F.P., Pyle, B.H., and Stewart, P.S. 1994 A minireview: Physiological assessment of bacteria using fluorochromes. *J. Microbiol. Meth.* [In Press]), and Rhodamine 123 has been applied successfully to the study of bacterial biofilms in our laboratory.

The confirmatory step with staining for microscopic examination can also be varied. For a rapid confirmation that cells have bound to the immunomagnetic particles, the rinsed particles can be stained with a general fluorochrome such as acridine orange or DAPI (McFeters et al., 1994). DAPI can be used in the same way as a counterstain after incubation with CTC. It is possible to follow the physiological activity incubation by treatment with a fluorescently labelled oligonucleotide probe with specificity for 16s rRNA of the target species. Many Gram-negative and some Gram-positive bacteria have been detected using fluorescent oligonucleotide probes (Amann, R., Ludwig, W., and Schleifer, K.-H. 1992 Identification and *in situ* detection of individual bacterial cells. *FEMS Microbiol. Lett.* 100:45-50). Probes for gene sequences which probe for Verotoxin or other toxins or specific cell constituents permit more specific conformation than that provided by fluorescent antibody techniques.

In an alternative embodiment, immunomagnetic beads with cells attached may be immobilized and incubated on a nutrient medium for a few hours to allow the attached target cells to form microcolonies. This pretreatment may be followed by visualization by immunofluorescence or fluorescent oligonucleotide probing, or by CTC incubation with either immunofluorescence or fluorescent oligonucleotide probe analysis.

EXAMPLE 4

In a preferred embodiment immunomagnetic beads which are coated with an antibody which specifically binds to a particular species of target bacteria are mixed with a liquid suspension which may contain the target bacteria. The sample suspension containing the beads is allowed to interact for up to an hour, with gentle agitation.

The tube containing the sample is placed in a magnetic separator which causes the magnetic beads to which target bacterial cells have attached to separate from the liquid suspension. The liquid suspension is aspirated out of the tube, leaving the beads with attached bacteria. The beads may be washed with appropriate solutions to remove loosely bound bacteria and other particles from the suspension.

The bacteria on the beads are mixed with a solution containing a tetrazolium compound which is taken up by respiring bacteria and reduced to insoluble formazan crystals by the cytochrome system. Following or simultaneous with incubation with the respiratory indicator, cells on the beads may be treated with a fluorescent stain or a specific fluorescent conjugated antibody. Specific fluorescent conjugated antibodies for target bacteria are known to those of skill in the art. (Bohlool, B.B. & E. Schmidt. 1980. The immunofluor-escence approach in microbial ecology. pp. 203-241 In Advances in Microbial Ecology, Volume 4, edited by M. Alexander (Plenum Press, New York)).

The specimen is then mounted for examination by epifluorescent microscopy. When appropriate excitation-/barrier/emission light filters are used, the formazan crystals fluoresce bright yellow/red in contrast with the fluorescent counterstain or immunofluorescent antibody. The cells may be enumerated and the number used to determine the number of respiring target cells in the original sample.

The method is applicable to the detection of respiring, viable bacteria of a particular target species in suspensions of food and in water, wastewater or sediment samples.

An advantage of the method lies in the combination of determining respiratory activity in conjunction with immunomagnetic capture and subsequent staining or fluorescent antibody confirmation. The immunomagnetic concentration step may be combined with membrane filtration to collect the beads and bacteria, thus precluding subsequent loss of bacteria from the beads during the respiratory incubation and final staining steps. Results would be obtained within 4-6 hours with limited labor input.

By means of immunofluorescence, bacteria in a mixture can be specifically labelled and thus individual types of bacteria can be detected qualitatively under a fluorescence microscope, also quantitatively with an image analysis system and then quantitatively in a flow cytometer.

For the determination by means of immuno-fluorescence, specific antibodies against the bacterial strains to be detected are produced and coupled to a fluorescence colored material. After incubation of the bacterial mixed culture with these antibodies, only the appropriate target bacteria of interest are fluorescently colored and can be detected under a fluorescence microscope.

The production of polyclonal or monoclonal antibodies which are needed for this technique takes place according to known processes which do not need to be further explained here. In principle, bacteria from a pure culture are killed, injected into an experimental animal (rabbit or mouse) and the antiserum is obtained therefrom or B-lymphocytes are isolated therefrom and used for the hybridoma technique.

Antibodies are selected on the basis of their specificity and utility as known to those of skill in the art. In the case where monoclonal antibodies have considerable cross-reactivities, this may be problematic for environmental samples and polyclonal antibodies may be used.

It is preferred that the magnetic beads used in the above method be Dynabeads®. Dynabeads® are uniform, superparamagnetic microspheres (2.8 microns in diameter) with affinity purified antibodies on their surface. When incubated with a sample, Dynabeads® bind their target bacterium forming a bacterium:magnetic bead complex. This complex is separated from the heterogenous sample by placing the sample tube in a magnetic holder (Dynal MPC®). For example, Microbiology Selective Enrichment Products Dynabeads®, anti-*E.coli 0157* Dynabeads®, anti-*Salmonella*, are designed for rapid, immunomagnetic selective enrichment of microorganisms directly from pre-enrichment broths.

The beads are rapid and simple to use, the protocol saves 24 hours of valuable testing time compared to conventional selective enrichment media. The beads have high sensitivity and detect as low as 100 organisms/ml of pre-enriched sample. The beads allow for complete detection of over 200 serotypes of *Salmonella* and both motile and non-motile strains of *E.coli 0157*. An antibody coating of different specificity could be used to capture other specific target bacteria. The beads are also efficient providing concentration and purification of the sample by immunomagnetic separation (IMS) improves bacterial isolations.

The beads are versatile and can be used for many different sample types. For example, the beads are convenient in that hand-held instrumentation provides convenience and ease of use. Only a magnet (Dynal MPC®) is required for separation of the beads. Magnetic beads

are flexible and can be conveniently combined with existing manual and automated detection methods (ELISA, PCR, and Impedance) for greater testing efficiency. The isolated and concentrated bacterium:bead complex can then be cultured on any selective culture medium or used in other detection systems. Dynabeads® anti-*E.coli 0157* are coated with affinity purified polyclonal anti-*E.coli 0157* antibodies. Dynabeads® anti-*Salmonella* are coated with affinity purified polyclonal and monoclonal anti-*Salmonella* antibodies. Beads can be coated with other antibodies by the suppliers or in the testing laboratory.

The method of the present invention can be used to obtain a viable enumeration of biological entities with a cytochrome system. It is preferred to use the present method to enumerate bacteria or protozoa.

Representative suitable means for measuring biological material, using naturally occurring properties of biological entities, or using stains, includes physical means such as optical, weighing, sedimentation, field flow sedimentation fractionation, acoustic, magnetic, electrical and thermal means. It is preferred to use optical measurements wherein biological material volumes are measured using optical phenomenon such as light scattering, light absorbance or calorimetric, fluorescence, time-delayed fluorescence, phosphorescence and chemiluminescence.

Optical measurements can be often enhanced by treating or exposing bacteria to at least one staining process, wherein at least one stain is utilized to enhance the measurement of biological material.

Representative suitable types of stains which may be used in addition to the fluorochrome for the detection of respiring bacteria, include stains indicative of biological composition, stains indicative of enzyme activity, and stains indicative of cell membrane integrity. Such stains are generally selected to have

readily measurable properties such as fluorescent stains, light absorbance stains and light scattering stains, and can be further selected according to the class of biological material which is stained, including, therefore, stains such as nucleic acid stains, protein stains, lipid stains, cell membrane stains, cell wall stains, stains responsive to enzyme activity, stains responsive to transmembrane potentials and cell surface receptor stains.

It is also useful to practice the present method with optical measurements made using apparatus such as flow cytometry apparatus, flow-through-microfluorimetry apparatus, optical particle analysis apparatus, fluorescence microscopy apparatus, light microscopy apparatus, image analysis apparatus and video recording apparatus.

Electrical measurements also have significant advantages, as electrical signals can be coupled directly to computational means. Thus, it is useful to practice this invention by employing electrical measurement means to measure biological material. Electrical measurements useful with bacteria include those involving electrical resistance particle analysis apparatus and dielectric property measurement apparatus and a dielectric property measurement apparatus.

For example, it is well established that a resistive cell counter, often termed a Coulter Counter, can use electrical resistance measurement to determine cell volume (see, for example, Kachel in Flow Cytometry and Sorting, Melamed et al (Eds), Wiley, New York, pp. 61-104).

In the case of bacterial cells stained by fluorescent compounds, optical analysis such as digital fluorescence microscopy or flow cytometry is used to analyze individual bacteria, using a wavelength band sufficiently different from that used for any detection

or measurement of bacterial properties so that simultaneous, or serial, measurement of bacterial properties is possible. The associated fluorescence signals are acquired and analyzed, with correction for spectral overlap if necessary, by conventional means.

The magnitude of the optical signal due to the cell stain in each bacterium, or small group of bacteria, is compared to the fluorescence of individual cells, thereby providing a calibration. Comparison of the bacterial signal magnitude to that of individual cells provides the basis for determination of growth of individual cells, for which the growth determination can often be made within about one generation time, but without a need for significant prior culture to obtain large numbers of cells, and growth can also be determined over several generations, if desired.

By making a large number of such individual cell growth determinations, the distribution of growth rate, distribution of lag time, and the plating efficiency caused by the exposure to one or more compounds or agents can be automatically determined by computer calculation. Other measurements relating to cell survival and cell death, particularly vital stains such as transmembrane potential stains, membrane exclusion stains and intracellular enzyme activity responsive stains, can also be used. Manual or visual inspection and scoring of bacteria can also be used, but is relatively labor intensive and therefore more prone to error. Thus, the preferred process is that conducted using the automated measurement means.

This invention can be used to provide measurement of certain types of biological entities or bacteria, herein referred to as analyte entities, capable of reacting with and binding two or more labeled specific binding molecules, wherein the labeled specific binding molecules are measured directly by measuring one or more

labels which have been attached to the individual labeled specific binding molecules, or are measured indirectly through the subsequent binding of additional, labeling molecules which can bind to, and thereby label, the labeled specific binding molecule. Examples of suitable specific binding molecules are antibodies, antigens, nucleic acids, avidin-biotin, enzyme inhibitors and lectins. A key property of analyte entities is that the analyte entities preferably have two or more specific binding sites which can bind labeled specific binding molecules.

The method of the present invention is generally applicable to many types of specific binding molecules. For example, the method of the invention is illustrated by considering an analyte entity with two distinct epitopes. In the case of labeled specific binding molecules, a sample containing the analyte entity is exposed to two different labeled antibodies, with one antibody specific for each of the two distinct epitopes. Following mixing, if desired, and after waiting for diffusion, encounter and binding of the labeled antibody molecules, the analyte entities have a high probability of being specifically labeled with two labels because of the binding of the two labeled antibodies.

One or more measurements of the amount of label in each bacterium is then made, such that the measurement is capable of resolving the difference of one label from two labels, and other measurements are made which allow the measurement of each bacterium. It is preferred to measure individual bacteria for volume and activity, but in some cases two or more bacteria can be measured together. Such measurement is used to characterize each bacterium.

The measurement process can involve a counting process, the invention provides means for measurements over a large dynamic range of analyte concentrations,

that is, from high concentrations to orders of magnitude lower concentrations.

Prior to the carrying out of the process of this invention, two or more labeled specific binding molecules are obtained, using means well known in the art, such that two or more labeled specific binding molecules are prepared, which are capable of binding to two or more binding sites on the analyte. Antibodies which bind to at least two non-overlapping epitopes on the analyte, such that at least two antibodies can be simultaneously and specifically bound to the analyte. Examples of such labeled specific binding molecules include (a) monoclonal antibodies with about one label molecule bound to each antibody molecule, (b) antigen molecules with about one label molecule bound to each antigen entity, (c) monoclonal antibodies with about two label molecules of the same type are bound to each antibody molecule, (d) antigen molecules with about two label molecules of the same type bound to each antigen entity, and (e) polyclonal antibodies containing at least two antibodies capable of binding to at least two non-overlapping epitopes of the analyte entity.

Analyte entities with at least two non-overlapping and non-competing specific binding sites can be measured. The important general class of analyte entities for bacteria include labeled antibodies, antigenic analyte entities capable of independently binding antibodies at two or more different sites can be measured. Examples of such analyte entities with two such sites include all antigens capable of assay by a sandwich assay.

In the general practice of this invention, analyte entities such as microorganisms with a cytochrome system can be measured.

SECTION II

At present, there is no rapid and accurate diagnostic test to detect VTEC in contaminated meat or clinical specimens. Unlike commensal (non-pathogenic) E. coli strains, VTEC must have two attributes to be pathogenic i.e., production of a Shiga-like toxin and the ability to adhere to intestinal epithelial cells. VTEC and other serotypes of E. coli that produce similar Shiga-like toxin II (6) are intestinal colonizers of farm animals. Transmission to man generally occurs through the consumption of contaminated and undercooked meat, especially ground beef. In some cases, infections may occur in day care centers, rest homes and in other institutional settings as a consequence of unsanitary conditions that predispose to transmission by many vectors in the environment.

Conventional diagnostic procedures including culture and serology are slow and costly. In the case of hemorrhagic colitis produced by VTEC, the serological confirmation of the causative agent is complex and expensive because other serogroups of E. coli have been implicated in this form of enteric disease in man. Wells et al. (7) found 8 percent of adult cows and 19 percent of heifers and calves positive for E. coli producing Shiga-like toxin with the isolates distributed among 19 serogroups including O157.

Willshaw et al. (8) found 48 strains of VTEC among 17 O serogroups other than O157 and O26. Moreover, antisera directed against O157 and H7 antigens may cross react with commensal E. coli or even distantly related genera (9). These results suggest that use of antisera to O somatic and H antigens of E. coli are not exclusive or inclusive diagnostic reagents for all verocytotoxic E. coli serogroups.

E. coli O157:H7 produces a distinctive cytotoxic action on Vero cells promoted by a Shiga-like toxin that has been shown to be encoded by a bacteriophage. Because

verocytotoxic strains of E. coli (VTEC) are not restricted to serogroup O157, there is need to develop a simple, but inclusive near term diagnostic test that quickly detects all strains of VTEC E. coli under field conditions.

The inventors have developed a unique method for the rapid detection of small numbers of specific viable bacteria, including E coli (O157:H7) and Salmonella typhimurium, in water systems. This innovative biomedical technology can be used for enhanced detection of VTEC in food, especially ground beef. VTEC bacteria are concentrated from suspensions of ground meat by immunomagnetic capture using superparamagnetic beads coated with monoclonal antibodies (mAbs) produced by hybridomas directed against novel surface or adhesin antigens believed to be required for attachment to intestinal epithelial cells and common to most VTEC. Bacteria attached to mAb-coated beads are incubated with the respiratory fluorochrome cyanoditolyl tetrazolium chloride (CTC) to enumerate viable organisms. The microscopic visualization of adherent, viable bacteria on the beads constitute a positive presumptive test for VTEC. The protocol includes a follow-on confirmatory assay employing fluorescent antibodies specific for Vero cytotoxin.

The products of the invention include a diagnostic kit and reagents that permits rapid enumeration of viable VTEC. Identification of the topology of the E. coli adhesin molecule with random peptide phage display analysis allows the development of highly specific antigenic preparations for both diagnostic as well as therapeutic uses.

The diagnostic kit may be used for quality control of processed meats and foodstuffs; assessment of handling and storage; testing for adequate cooking; and future application as a prototype for detection of pathogenic

bacteria in water and sludges and as a diagnostic clinical test for other enteric pathogens.

Shear and static analyses of adhesin events. The inventors pioneered the development of both an in vivo and in vitro shear system capable of analyzing cellular and molecular events in which adhesion plays an important role. The in vitro shear analytic system examines cell-cell or microbe-mammalian cell interactions under physiological shear in capillary tubes which serves to mimic the intravascular environment. The in vivo shear model employs intravital microscopy to examine cellular interactions under physiological shear forces found in the venules of the intact animal. For both host and microbial adhesion to target cells, a static (in vitro) adhesion assay can also be conducted on tissue sections or tissue culture preparations of established cell lines or cells from any organ system. Both in vitro systems are adaptable to studies of adhesion events involving either bacteria, fungi or parasites with putative target cells from man or animals.

The inventors have developed a diagnostic test for the rapid detection and identification of E. coli O157 and Salmonella typhimurium in water systems (1). Their test system serves as a prototype diagnostic assay for the detection and identification of pathogenic bacteria associated with enteric diseases of man and animals with special emphasis on Escherichia coli (O157:H7) and other VTEC.

In addition, a method for the preparation of cell surface antigens (adhesins) from Salmonella typhimurium (2) is known. These same methods are applied to the extraction and purification of E. coli adhesins and the development of hybridomas for monoclonal antibody production.

The inventors have devised a rapid Presumptive Test by integrating a two step methodology including in

stepwise fashion (a) the immunocapture and enumeration of adhesin-positive (presumptively pathogenic) E. coli from meat homogenates and (b) the determination of the viability of target organisms with the respiratory fluorochrome CTC. The sensitivity of the immunoassay is 10 to 100 colony forming units (CFU) per gram of meat. The protocol also includes a fluorescent antibody test to confirm VTEC detection using anti-verotoxin antibody. Serogrouping of bead-bound organisms employs fluorescent labelled antisera against O somatic and H flagellar antigens to identify the specific serogroup of E. coli for tracking and reporting.

A approach to rapid and accurate detection and identification of VTEC resides with an immunoassay that identifies a universal or common antigen shared by all VTEC. In this regard, most VTEC appear to have common structures (adhesins) in their outer membrane other than pili that mediate cell adhesion to the epithelial cells of the intestine (10). The assumption that the structure of adhesins is restricted and, therefore, antigenically homologous is supported by the observation that homologous sequences to an eaeA gene probe from enteropathogenic E. coli (EPEC) have been found in VTEC (11). Moreover, intestinal epithelium is believed to have a common receptor molecule having a narrow specificity for or reactive with a single or a closely related bacterial adhesin. By defining adhesin topology, their role and mode of action in promoting adherence and colonization on the intestinal epithelium of the intestinal tract and provoking a host response to their presentation allows for therapeutic and preventive strategies to be formulated.

EXAMPLE 5

Cell adhesion: Junkins and Doyle (10) found that cell adhesion by E. coli O157 was influenced by growth

conditions provided for the bacteria. We are establishing the growth conditions required by our selected strain for maximum cell adhesion, evaluating especially: (i) oxygen tension, (ii) growth stage, (iii) medium, and (iv) temperature.

The method for measuring cell adherence in previous work with Salmonella (2) are used for detecting cell adhesion by VTEC. Briefly, bacteria from broth culture are diluted directly in tissue culture medium. Infected HEp-2 cell monolayers in tissue culture plates are centrifuged (500xg, 30 min) and washed immediately 5 times with Dublecco's phosphate-buffered saline (PBS), each wash including two min on a rotary shaker followed by aspiration to remove unassociated bacteria. The washed cells are lysed and the bacteria suspended by adding N-laurylsarcosine (0.5%) in saline with vigorous shaking for 5 min. The recovered bacteria, composited from triplicate monolayers, are counted by agar plate colony counts, and the degree of cell adhesion expressed as a percent of the inoculum, also counted at the time of addition to the cell monolayers.

Antigen Preparation:

A selected strain of VTEC are grown in broth cultures under conditions previously identified as optimum for expression of maximum cell adhesion. The bacteria are recovered by centrifugation, washed in PBS, and biotinylated in 50mM sodium bicarbonate (pH 8.5) containing sulfo-NHS-biotin. The recovered bacteria are incubated on a roller, recovered by centrifugation, washed in PBS, and then dialyzed to remove free biotin.

The procedure requires an antigen preparation from the bacteria that is not harmful to cells in tissue cultures. When this procedure was worked out for Salmonella, the antigen preparation was a water-extract

of biotinylated bacteria, which, after buffering at pH 7.2, presented no problem when added to cell cultures. However, it is unlikely that a simple water extract is going to recover all of the antigens of interest from VTEC, and that complete recovery requires extraction with detergents, which are inimical to animal cells in even trace amounts. The detergents selected for extraction of the bacteria must, therefore, are removed before addition of the extract to the cells, and this can be accomplished by selecting detergents that are dialyzable.

Extracts are prepared from the biotinylated bacteria using 1% sodium desoxycholate, n-octyl-ß-D-thiogluco-pyranoside or 2M urea. Centrifugation, dialysis, and ultrafiltration are used to purify and concentrate these extracts. Dot-blot procedures, using avidin-HRP (horseradish peroxidase) as the probe and 4-chloronapthol (4-CN) as the enzyme substrate, are used to confirm the presence of biotinylated proteins. Extracts containing biotinylated proteins are added to washed HEp-2 cell monolayers, incubated (37oC for 2 h), washed to remove all unassociated biotinylated proteins. Cells are lysed then extracted for 10 min, and DNase added to solubilize viscous DNA. The presence of biotinylated proteins are confirmed by dot blot assay. Finally, the biotinylated proteins in the cell extract are adsorbed onto avidin-sepharose beads, centrifuged gently, and washed to remove unadsorbed material. These beads should carry those bacterial proteins that adhered to the cells, i.e., the cell adhesion-mediating proteins from the bacteria. These proteins are referred to as adhesins or Cell Adhesion Mediating (CAM) proteins.

EXAMPLE 6

The inventors have been studying cell adhesion and invasion by enteropathogenic bacteria for nearly 17 years, beginning with Yersinia enterocolitica (21,22) and more recently with Salmonella typhimurium (23,24). They have developed a novel technique for recovering cell-adhesion mediating antigens from bacteria that permits the preparation of specific monoclonal antibodies (2).

Development of hybridomas and monoclonal antibodies (mAbs) against cell adhesion-mediating (CAM) proteins (Adhesins).

Anti-adhesin mAb hybridomas are prepared in accord with well established techniques reported in prior publications (15, 16) and screened by ELISA. In all cases, BALB/c mice, ranging in age from 6 to 12 wk, are used for hybridoma and antibody production. The mice are housed in the MSU small-animal facility and all procedures conducted.

Immunization protocol: Primary immunization antigens (adhesins) are adjusted to 20 g/dose in .5ml PBS and emulsify in CytRx TiterMax #R-1 at a 50/50 ratio. Immunized mice are bled at Day 14 to test antibody responses by ELISA. If a low titer is found the mice are boosted with 10-20 g of antigen and bleed and tested at Day 28. This procedure is repeated at 2 week intervals until the desired antibody titers for anti-adhesin antigens are achieved.

ELISA screening protocol:

ELISA assays employ Costar universal covalent surface immuno assay plates and standard protocols for binding of the bacterial adhesins. The system employs a two or three stage development system in which the

adhesin antigen are covalently bound to the 96 well assay plates by UV crosslinking. The 1st stage anti-adhesin specific mouse serum or hybridoma supernatant antibodies are diluted and incubated in the plate wells after the adhesins are bound and the plates are blocked with BSA. After washing to remove residual 1st stage, a peroxidase coupled anti-mouse 2nd stage antibody are added to the test wells, incubated, and washed.

Substrate is then added to the wells to develop the assay which is subsequently be read by an automated ELISA plate reader. If the desired sensitivity has not been achieved, a three stage amplification is employed.

Hybridoma fusion protocol:

Day -3: Prior to the final immunization, the spleen donor are bled to obtain serum for testing for specific antibody titers against adhesins by ELISA. The mouse is then be boosted with antigen in saline I.V.. If the antigen is not in a form that is injectable I.V., it are injected I.P. in saline and the interval before fusing are four days. Day 0: The fusion are completed as follows: 1) Count and harvest mouse myeloma cells for fusing with the mouse spleen cells. 2) Sacrifice the spleen donor and bleed the mouse for serum, prior to removing the spleen, by perfusion with sterile saline to obtain the maximum blood volume. 3) Remove the spleen aseptically and remove any connective tissue. 4) Make a spleen cell suspension, filter and centrifuge. 5) Combine the SP2/0 and spleen cells in one centrifuge tube and wash by centrifugation. 6) Aspirate the medium and add PEG and gently resuspend the cell pellet. Centrifuge and carefully aspirate off the PEG and add medium to resuspend the pellet; centrifuge and aspirate off the medium. 7) Immediately, gently resuspend the pellet in HAT medium and dilute to the desired total plating volume plating as soon as practical and place the

plates in the CO2 incubator. Day 5: Fusion wells are fed with HAT medium in volumes equal to the original plating volume. Day 7-14: The screening and selection of hybridoma clones is completed as follows: 1) Examine clone growth to plan and anticipate the day for screening of hybridomas. 2) When clones have grown to sufficient size, pull supernatants for testing and refeed. 3) After selection of desired clones by ELISA screening expand cell numbers for subcloning and for freezing.

Anti-adhesin hybridomas are subcloned and selected: 1) As soon as hybridoma cell numbers permit, proceed with 96 well plate subcloning by limiting dilution in HAT medium. 2) Upon successful ELISA screening of mAb positive subclones the hybridomas are adapted to serum free medium for bulk production of anti-adhesin mAb.

EXAMPLE 7

Plain immunomagnetic beads are obtained commercially, e.g. from Dynal, and coated with adhesin mAb using a protocol recommended by Dynal. In essence, the beads are mixed with an appropriate amount of mAb for 30 min at 4C, collected in a magnetic particle concentrator, washed 5 times and finally suspended in buffer. The coated beads are incubated with suspensions of the VTEC from which the adhesin was extracted, and at least 4 other strains of VTEC, to determine the efficiency of capture of target organisms. Cells attached to the beads are stained with DAPI and examined using fluorescence microscopy to determine the number of attached cells. After magnetic separation, supernatants are passed through 0.2 m porosity black polycarbonate membrane filters, stained with DAPI and cells enumerated by epifluorescence microscopy. This permits an assessment of the reliability of microscopy for

enumeration of cells on beads, as well as the efficiency of cell capture.

Presumptive Test

Ground beef samples artificially inoculated with VTEC are added to a suitable extraction medium. Following brief (5-10 min) processing in a Stomacher, the homogenate are transferred to a tube and a suspension of mAb-coated magnetic beads added. The homogenate are incubated with the beads for 10-60 min, then placed in a magnetic particle concentrator to immobilize the beads and captured cells. Anti-E. coli O157 Dynabeads are used for comparison. Commercial antibodies prepared against E. coli O157:H7 or other strains of VTEC may also be used to coat beads and evaluated for isolation of VTEC. Other bacteria, including non-VTEC, are used to confirm the specificity of the anti-adhesin-coated beads.

After 1-5 min, the liquid meat/buffer suspension are removed by aspiration. The tube are taken off the magnetic separator and the beads resuspended in fresh buffer and membrane filtered, followed by either DAPI staining or incubation with fluorescently labeled E. coli O157 antibody, rinsing, and mounting for epifluorescent microscopic examination and counting (Figure 7). Procedures developed for fluorescent antibody staining on membrane filters are employed.

Variations in this procedure are introduced in order to optimize the detection of VTEC. These include diluents, meat:diluent ratios, incubation times, temperatures and procedures for the meat plus bead suspensions, bead recovery, and washing methods. If it is found that direct microscopic examination of the beads is inefficient for visualizing all of the attached cells, procedures for removal of bacteria from the beads, e.g. incubation with a low pH glycine buffer as used for

antibody purification, are employed prior to filtration, staining and microscopic examination.

For situations where it is important to determine the viability of VTEC, cells captured on immunomagnetic beads are incubated with the respiratory fluorochrome cyanoditolyl tetrazolium chloride (CTC) prior to the confirmatory tests. Beads with captured bacteria are incubated with the respiratory fluorochrome cyanoditolyl tetrazolium chloride (CTC) before counterstaining with DAPI or reaction with FA preparations. A preparation of E. coli O157:H7 incubated with CTC followed by FA staining is shown in Figure 8.

Following immunomagnetic separation, fluorescent antibodies to Vero cytotoxin (30) are used to confirm the detection of VTEC by staining and epifluorescent microscopy. Additionally, for confirmation of specific serogroups, such as O157, fluorescent labelled antibodies are obtained commercially, e.g. from Kirkegaard and Perry Laboratories or Biodesign Inc. For VTEC serogroups other than O157, if commercially conjugated antibodies are unavailable, unlabeled antibody preparations to other VTEC serogroups are obtained and conjugated with appropriate fluorochromes, e.g. FITC or TRITC.

Commercially available enzyme-linked immunosorbent assay systems, e.g. EHEC-Tek (Organon Tecknika Corp.), as used following immunomagnetic separation by Johnson et al (1995) are utilized.

Cells stained green have reacted with the fluorescein- labeled antibody. Those with an orange/red spot in them have reduced the CTC to CTC formazan which indicates respiratory activity.

Incubation with CTC to detect respiratory activity are compatible with immunomagnetic capture and subsequent fluorescent antibody confirmation.

EXAMPLE 8

Using ground beef samples obtained from local supermarkets, the proposed method are evaluated to determine a number of performance characteristics of assays. Separate ground beef samples are inoculated with VTEC cultures. Five to ten levels of inoculum are used, ranging from <1 through ca. 500 CFU/25 g. Uninoculated control samples are assayed to detect inherent contamination with these pathogens and the levels of nontarget bacterial contaminants. Uninoculated and inoculated sterile meat such as canned minced beef dog food, are used for negative control tests and to detect possible interferences from meat constituents. All experiments are performed at least three times to permit statistical comparison. The method performance characteristics include: Recovery, Calibration Curve, Limit of Detection, Limit of Quantitation, Sensitivity and Specificity.

a. Recovery: Recovery are reported as a percentage, based on the number of cells detected versus the number inoculated. Three different levels of inoculation are used, over the range of <1 through ca. 500 CFU/25g.

b. Calibration Curve: Calibration curves are developed by comparison of results from the proposed simple, rapid method with the best culture method, e.g. enrichment techniques, such as lauryl sulfate tryptose broth (LST) and EC broth containing novobiocin. These data also provides an estimate of linearity.

c. Limits of Detection: The limits of detection and quantitation are determined from the recovery and calibration curves.

d. Sensitivity and Specificity: Sensitivity and specificity are calculated from the numbers of true positive, true negative, false positive, and false negative results, based on the performance of positive and negative control tests. Inoculated ground beef samples are used for positive control tests, using both

the target organism and also some non-target species. The non-target species include a Salmonella sp. other than S. typhimurium, and Klebsiella pneumoniae. Canned minced dog food inoculated with nontarget species are used for the negative control samples.

Epitope mapping and Peptide Determination

Epitope mapping using random phage-display libraries offers a tool to aid in determining key molecular structures of glycoproteins embedded in the complex topologies of procaryotic or eucaryotic cell membranes (26). With the use of a monoclonal antibody (mAb) to provide a template of the target epitope topology, a bacteriophage peptide-display library containing as many as 5 x 108 unique nonapeptide sequences may be probed by the specific mAb via binding of phage displaying a specific consensus peptide sequence. Where mAbs recognize linear epitopes the approach may be universally applicable. In those situations where tertiary structure defines the epitope, the mAb may recognize portions of the critical amino acid sequence or side chains with an efficiency well enough to determine and reconstruct the reactive site for diagnostic purposes.

Three cycles of immuno affinity purification and amplification are typically used to select and narrow the specificity of phage displaying peptides bound by the mAb. The unique peptide sequences of specifically bound phage are then determined by sequencing the phage DNAs coding for the peptides. The synthesis of the peptide sequence and subsequent conformation of the epitope topography is determined by its reactivity with the mAb in a Western Blot analysis.

The inventors develop and use methods that precisely define adhesion events involved in the infectious process. In addition to analyses of cellular and molecular components of an inflammatory reaction

associated with infections, inventors analyze specific adhesion events in vitro that define the role of bacterial adhesins in attaching to specific receptors on intestinal and endothelial cell membranes under physiological conditions (18).

Questions have been raised about the specificity of existing E. coli O157:H7 antibodies. The advantage of the proposed method is that two antibodies can be used, one for the initial capture of the target bacteria on the immunomagnetic beads, and the other for confirmation after the incubation for respiratory activity. Thus, if a broad-spectrum antibody which reacts with a common antigen (adhesin) shared by most VTEC is used to coat immunomagnetic beads, this would help to optimize detection. A more specific fluorescent antigen could then be used for confirmation.

The objective is to detect less than 10 colony forming units per gram of meat is expected, it has been found that levels of 10-6200 E. coli O157:H7 per gram have been detected in beef samples associated with food-borne illness (Todd et al., 1988). Thus, a rapid, specific method which can detect 10 viable VTEC per gram of sample is useful in the identification of meats which are likely to cause food-borne disease.

The invention uses an efficient capture mechanism employing magnetic beads coated with a monoclonal antibody reactive to adhesins or intimin epitopes believed to be common to most VTEC. A antibody to Vero cytotoxin are used to confirm the detection of VTEC.

Aspects of the present invention include protocols for the tests on various food sources; monoclonal antibodies directed against common surface antigens or adhesins of VTEC and related serogroups; purified adhesin antigens; fluorescent labeled antibodies and superparamagnetic beads coated with the monoclonal antibodies.

The applications of the diagnostic kit for detecting contamination of food and water with VTEC bacteria are extensive. Use of the method has enormous impact both in the food production industry and in terms of public health. It is applicable to: 1) Quality control during processing and storage of meats and other foodstuffs; 2) Detection and assessment of handling and storage problems; 3) Confirmation of adequate cooking; 4) Diagnostic methods for detecting other enteric pathogens; 5) Broad applications extending to the detection of pathogenic bacteria in water, sludges and sediments as well as in the clinical laboratory setting. The method of the invention reduces the cost of testing, provide simplicity for minimally skilled personnel, provide reliability and repeatability of test results, and detect 10 to 100 organisms per gram of specimens.

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The purpose of the above description and examples is to illustrate some embodiments of the present invention without implying any limitation. It will be apparent to those of skill in the art that various modifications and variations may be made to the composition and method of the present invention without departing from the spirit or scope of the invention. All patents and publications cited herein are hereby incorporated by reference.

WE CLAIM:

1. A rapid method for the detection, identification and enumeration of respiring microorganisms comprising the steps of

a) passing a microbial sample through a collecting device to capture the cells;

b) adding to the collecting device a fluorochrome dye specific for the detection of respiring cells and allowing the dye to incubate;

c) treating the collecting device with a reactive fluorescent antibody which reacts with a microorganism of interest present in said microbial sample;

d) mounting said collecting device for examination by fluorescence microscopy in which a suitable light system is used to excite the fluorochrome dye and fluorescent antibody to fluoresce; and

e) quantifying said respiring target microbial cells.

2. The method of claim 1, wherein said collecting device is selected from the group consisting of a black polycarbonate membrane filter, a reverse flow filter, sedimentation field flow fractionation, and immunomagnetic beads.

3. The method of claim 1, wherein said collecting device is selected from the group consisting of any non-fluorescing filter, a reverse flow or tangential flow filter, an immunocapture device and centrifugation techniques.

4. A rapid method for the detection, identification and enumeration of respiring microbes comprising the steps of

a) filtering a sample through a membrane filter to capture microorganisms;

b) transferring the membrane filter to a pad comprising a fluorochrome dye specific for the detection of respiring cells;

c) incubating said sample with said fluorochrome dye,

d) treating the surface of the membrane filter with a reactive fluorochrome labeled antibody which reacts with a specific microbial species or strain of interest present in said sample;

e) mounting said membrane filter for examination by fluorescence microscopy in which a suitable light filter system is used to excite the fluorochrome dye and fluorochrome labeled antibody to fluoresce; and

f) quantifying said respiring target microbial cells.

5. The method of claim 4, wherein said fluorochrome dye specific for the detection of respiring cells is a tetrazolium compound.

6. The method of claim 4 wherein said fluorochrome dye specific for the detection of respiring microbes is taken up by respiring cells and reduced to insoluble formazan crystals by the cytochrome system of said cells.

7. The method of claim 5, wherein said tetrazolium compound for the detection of respiring microbial cells is 5-Cyano-2,3-Ditolyl Tetrazolium Chloride (CTC).

8. The method of claim 4, further comprising a step of rinsing the membrane filter to remove unbound fluorochrome labeled antibody after step d.

9. The method of claim 4, wherein said fluorescence microscopy is epifluorescence microscopy.

10. The method of claim 4, wherein said membrane filter is a black polycarbonate membrane filter.

11. The method of claim 4, further comprising a gelatin blocking step to minimize non-specific antibody binding.

12. A rapid method for the detection, identification and enumeration of respiring microorganisms comprising the steps of

a) mixing immunomagnetic beads comprising an antibody which specifically binds to microbial cells with a liquid sample comprising said microbial cells;

b) allowing said liquid sample to interact with the beads for up to an hour;

c) placing the sample in a magnetic separator which causes the magnetic beads to which cells have attached to separate from the liquid sample;

d) aspirating the sample, leaving the beads with cells attached;

e) washing the beads with a solution which removes loosely bound cells and other particles from the liquid sample;

f) mixing beads with cells attached with a fluorochrome dye specific for the detection of respiring bacteria;

g) treating bacteria on the beads with a fluorescent stain or a specific fluorescent conjugated antibody;

h) mounting said sample for examination by epifluorescent microscopy, in which a suitable light filter system is used to excite the fluorochrome dye and fluorochrome labeled antibody to fluoresce; and

i) quantifying said respiring microbes.

13. The method of claim 12, wherein said fluorochrome dye specific for the detection of respiring microorganisms is a tetrazolium compound.

14. The method of claim 12, wherein said fluorochrome dye specific for the detection of respiring microorganisms is taken up by respiring cells and reduced to insoluble formazan crystals by the metabolism of said cells.

15. The method of claim 12, wherein said fluorochrome for the detection of respiring microorganisms is 5-Cyano-2,3-Ditolyl tetrazolium Chloride (CTC).

16. The method of claim 12, wherein step b is performed with gentle agitation.

17. The method of claim 12, wherein said method is automated with the use of robotics, microscopic image analysis, or flow cytometry.

18. The method of claim 12 wherein said collecting device to capture the microbial cells from a contaminated sample comprises a centrifuge for sedimentation field flow fractionation.

19. The method of claim 1 wherein said respiring microrganisms are selected from the group consisting of verocytotoxic E. coli (VTEC) and E. coli 0157:H7.

20. The method of claim 12, wherein said immunomagnetic beads comprise a monoclonal antibody against an antigenic determinant of a surface adhesin common to multiple verocytotoxic E. coli strains.

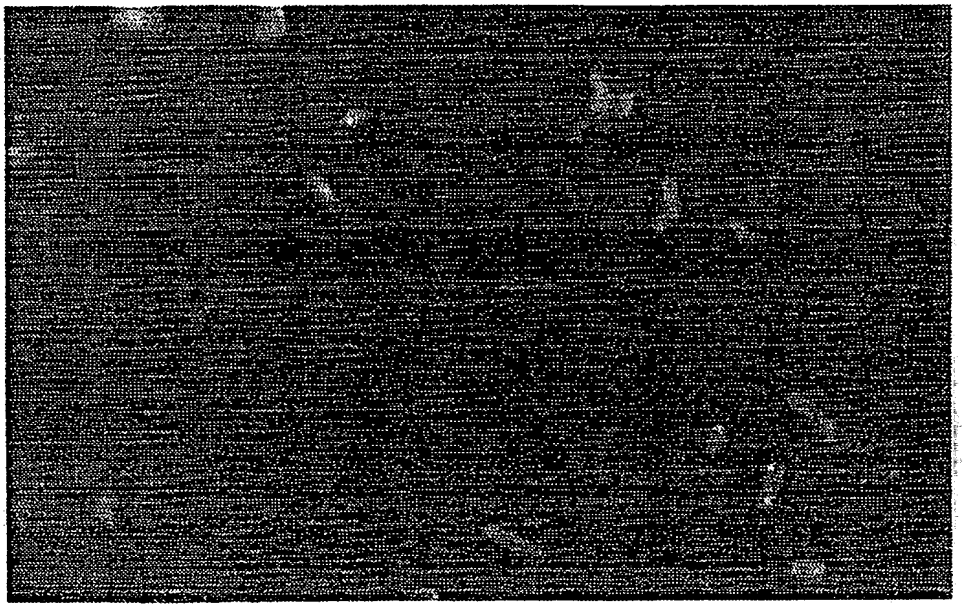
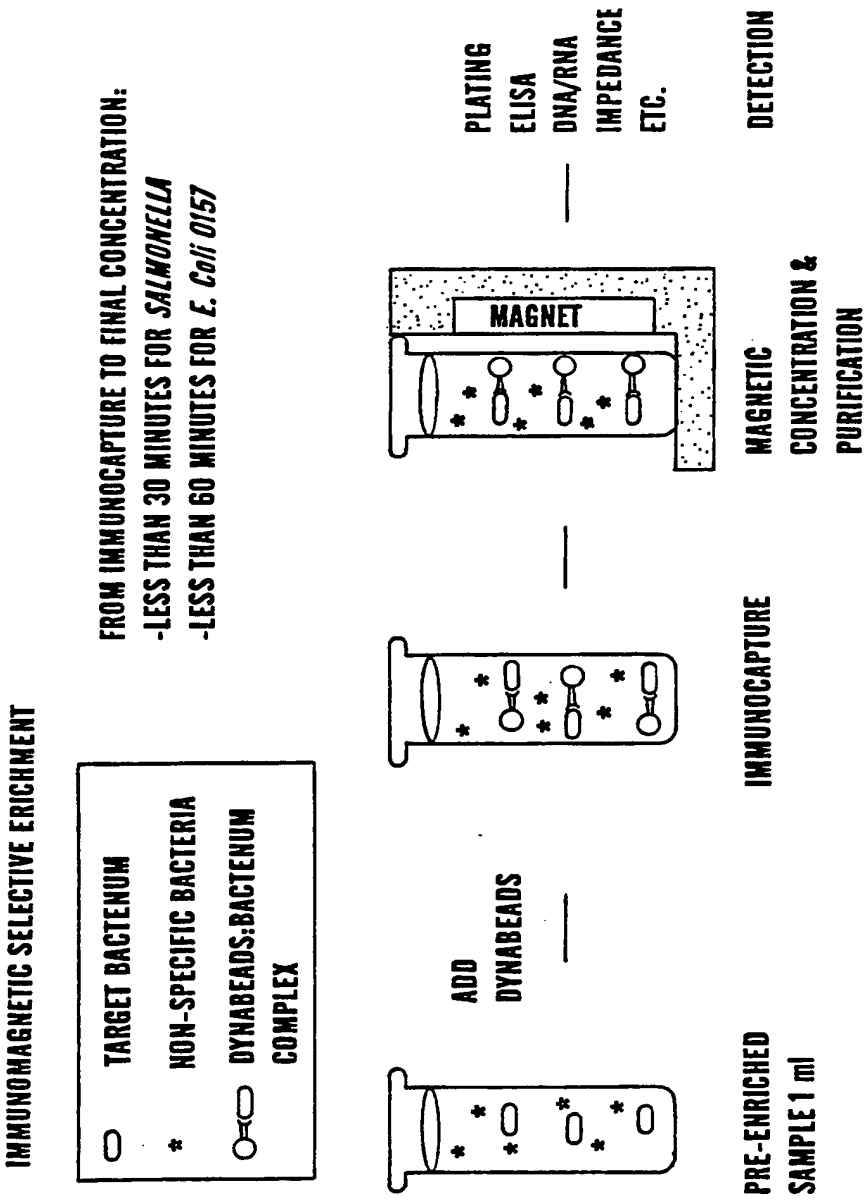


FIG. 1

FIG. 2



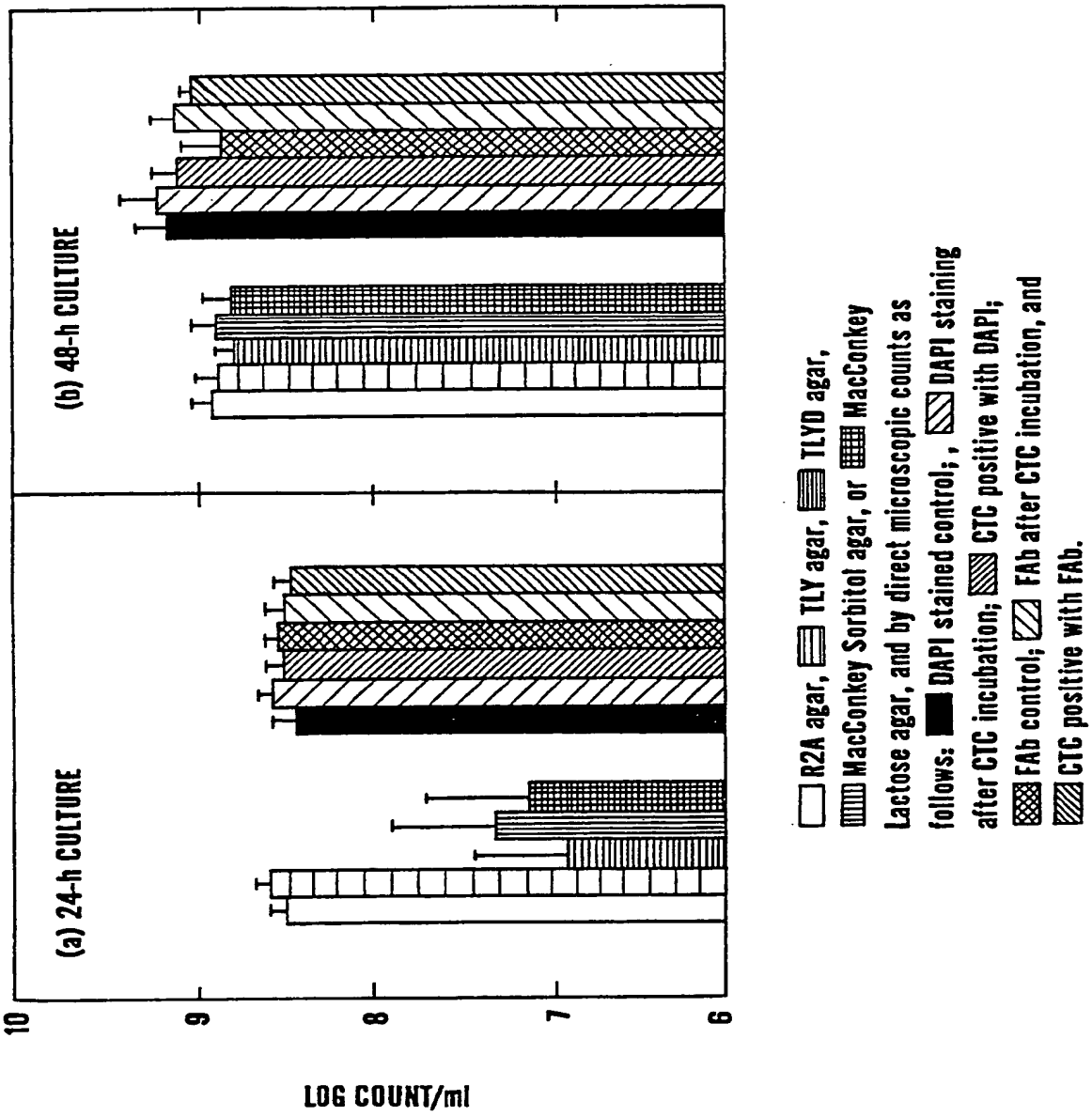

(a) 24-h CULTURE
(b) 48-h CULTURE
10
9
8
7
6
LOG COUNT/ml
R2A agar, TLY agar, TLYD agar,
MacConkey Sorbitol agar, or MacConkey
Lactose agar, and by direct microscopic counts as
follows: DAPI stained control; , DAPI staining
after CTC incubation; CTC positive with DAPI;
FAb control; FAb after CTC incubation, and
CTC positive with FAb.

FIG. 3

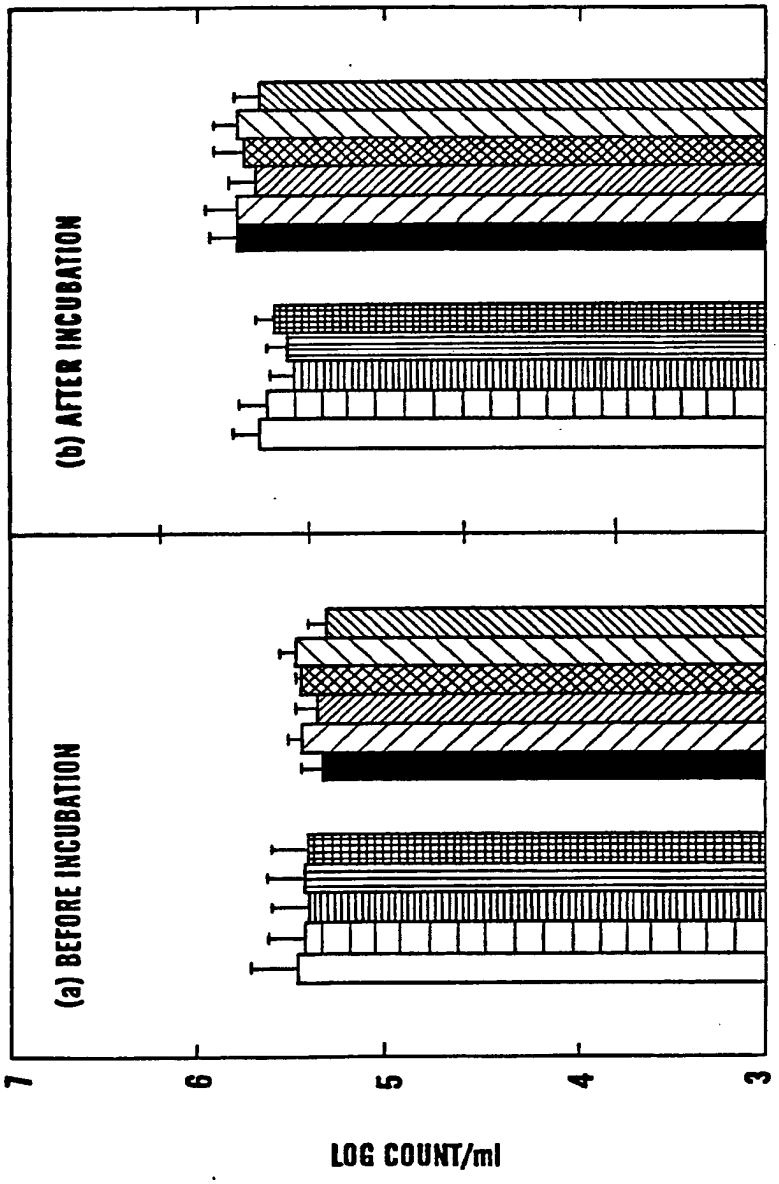

(a) BEFORE INCUBATION
(b) AFTER INCUBATION
7
6
5
4
3
LOG COUNT/ml
R2A agar, TLY agar, TLYD agar,
MacConkey Sorbitol agar, or MacConkey
Lactose agar, and by direct microscopic counts as
follows; DAPI stained control;, DAPI staining
after CTC incubation; CTC positive with DAPI;
FAb control; FAb after CTC incubation, and
CTC positive with FAb.

FIG. 4

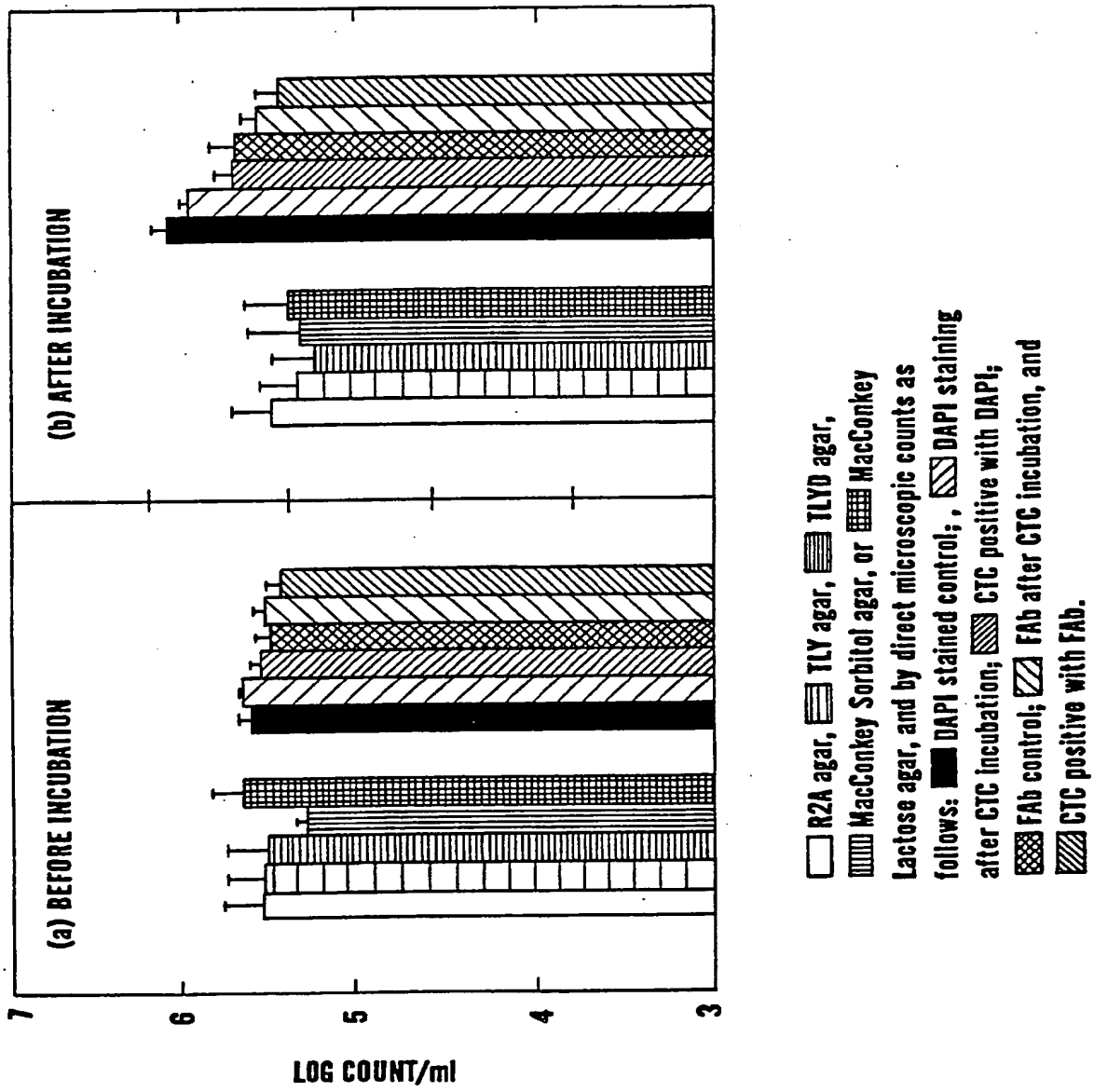

(a) BEFORE INCUBATION
(b) AFTER INCUBATION
7
6
5
4
3
LOG COUNT/ml
R2A agar, TLY agar, TLYD agar,
MacConkey Sorbitol agar, or MacConkey
Lactose agar, and by direct microscopic counts as
follows: DAPI stained control;, DAPI staining
after CTC incubation; CTC positive with DAPI;
FAb control; FAb after CTC incubation, and
CTC positive with FAb.

FIG. 5

FIG. 6

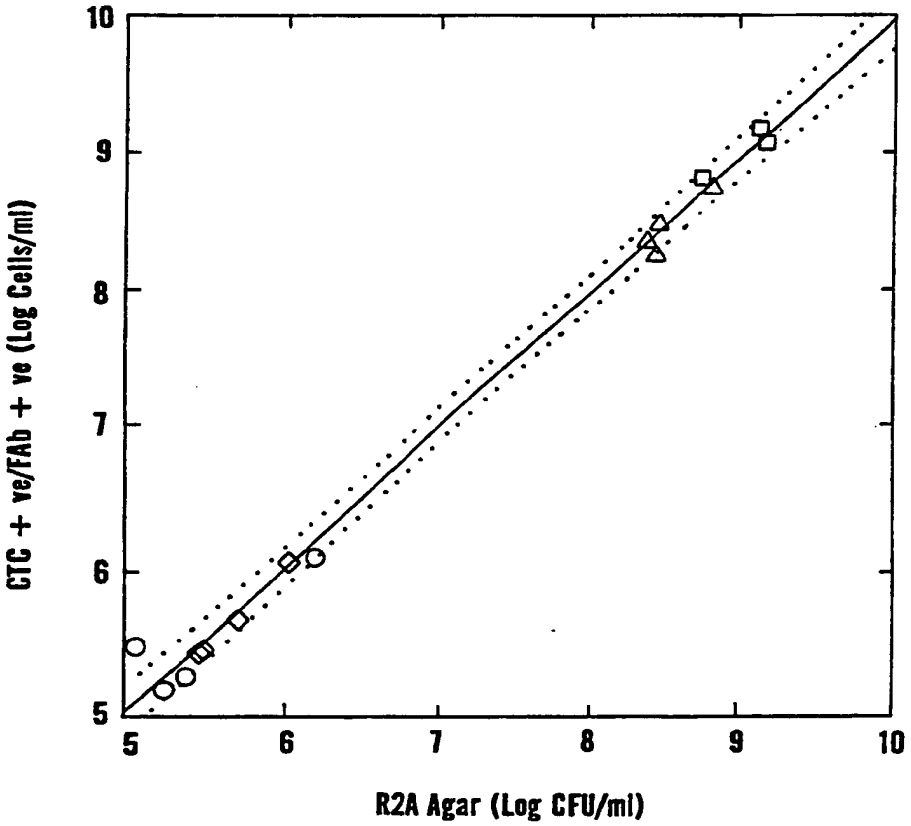


FIG. 7

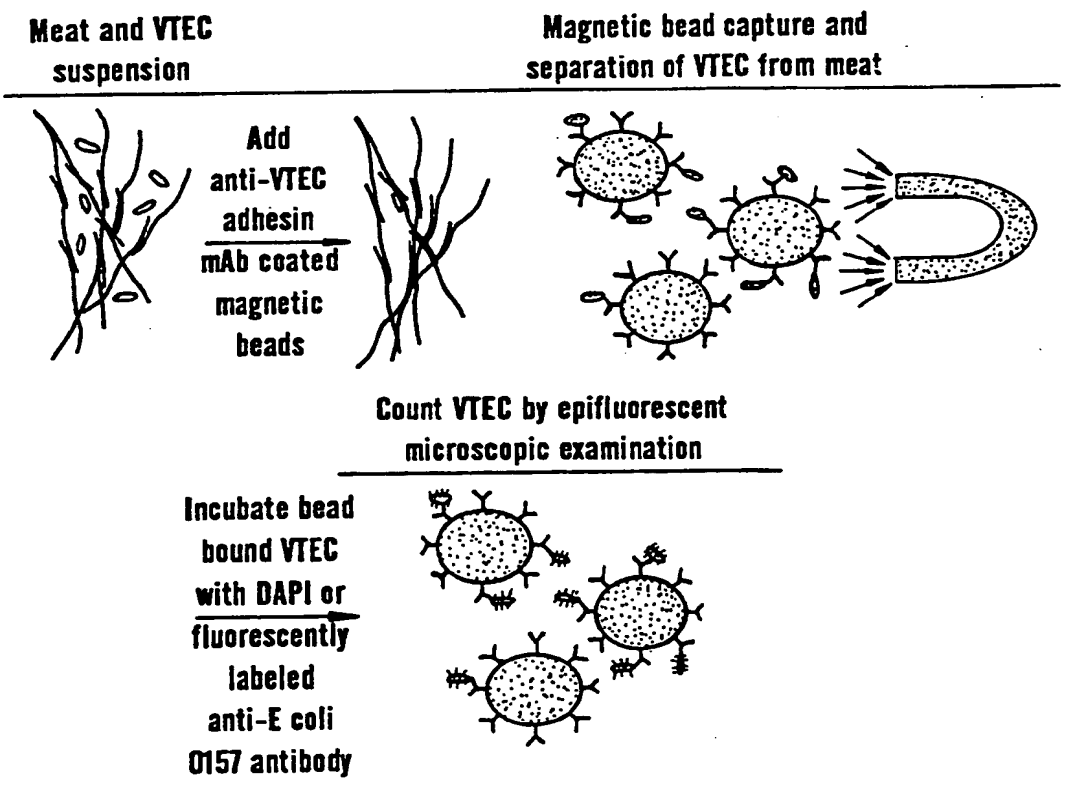
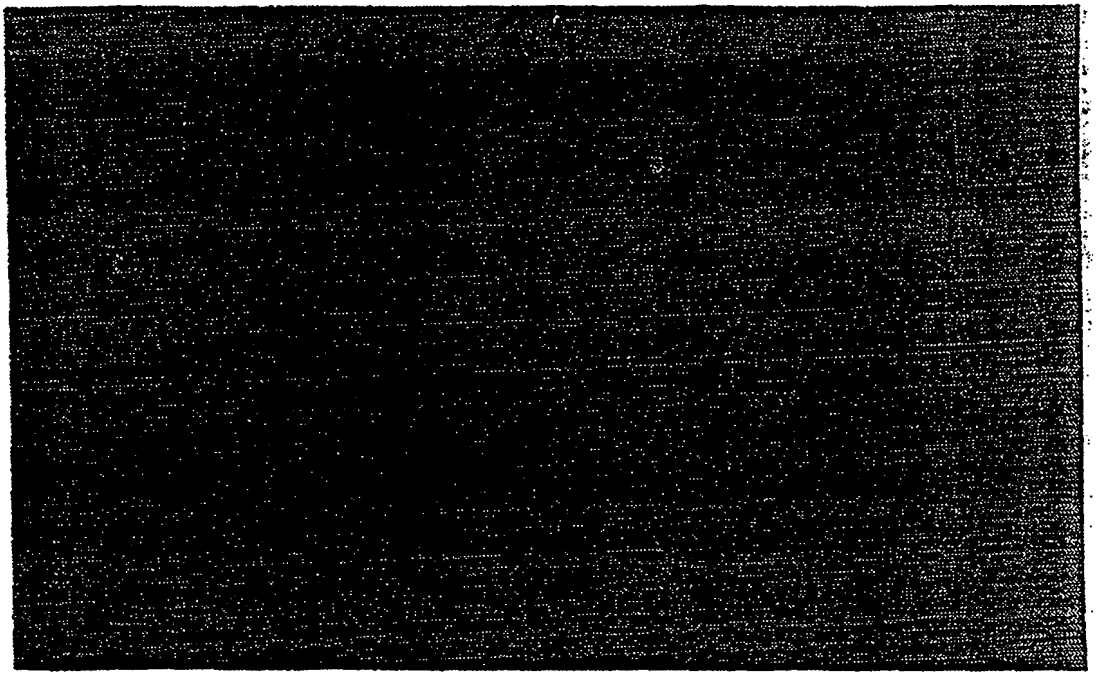

Meat and VTEC suspension
Add anti-VTEC adhesin mAb coated magnetic beads
Magnetic bead capture and separation of VTEC from meat
Count VTEC by epifluorescent microscopic examination
Incubate bead bound VTEC with DAPI or fluorescently labeled anti-E coli 0157 antibody

FIGURE 8: Photomicrograph of cells of *E. coli* O157:H7 reacted with rabbit anti-O157 primary antiserum and with goat anti-rabbit fluorescein conjugate after incubation with CTC*.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05971

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/7.2, 29, 174, 176, 803, 810; 436/525; 530/391.1, 391.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 29, 174, 176, 803, 810; 436/525; 530/391.1, 391.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSYS, CAB ABSTRACTS, EMBASE, DERWENT WPI
search terms: inventor(s), bacteria, microorgainsm, viable, live, respir?, enumerate, count, identify, detect, antibody, immunoglobulin, fluorescent, device, filtrat?, and metaboliz?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Applied and Environmental Microbiology, Volume 59, Number 11, issued November 1993, Schaule et al, "Use of 5-Cyano-2,3-Ditolyl Tetrazolium Chloride for Quantifying Planktonic and Sessile Respiring Bacteria in Drinking Water", pages 3850-3857, see entire document.	1-20
Y	Applied and Environmental Microbiology, Volume 58, Number 6, issued June 1992, Rodriguez et al, "Use of a Fluroescent Redox Probe for Direct Visualization of Actively Respiring Bacteria", pages 1801-1808, see entire document.	1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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14 AUGUST 1995	15 SEP 1995
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05971

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Water Research, Volume 22, Number 8, issued August 1988, Cloete et al, "A combined membrane filter-immunofluorescent technique for the in situ identification and enumeration of acinetobacter in activated sludge", pages 961-970, see entire document.	1-20
Y	Journal of Microbiological Methods, Volume 16, Number 3, issued 1992, Desmonts et al, "An improved filter method for direct viable count of Salmonella in seawater", pages 195-201, see entrire document.	2 and 10
Y	D. M. WEIR et al, "Handbook of Experimental Immunology In Four Volumes, Volume 1:Immunochemistry" published 1986 by Blackwell Scientific Publications (MA), pages 27.1-27.20, see especially page 27.6 column 1, first full paragraph.	11
Y	Eur. J. Clin. Micribiol. Infect. Dis., Volume 12, Number 1, issued January 1993, Islam et al, "Rapid detection of Shigella dysenteriae and Shigella flexneri in faeces by an immunomagnetic assay with monoclonal antibodies", pages 25-32, see entire document.	2, 3 and 12-18
Y	FEMS Microbiology Ecology, Volume 14, Number 3, issued 1994, Diaper et al, "Flow cytometric detection of viable bacteria in compost", pages 213-220, see entire document.	17
Y	Journal of Microbiological Methods, Volume 10, issued 1989, Singh et al, "Rapid enumeration of viable bacteria by image analysis", pages 91-101, see entire document.	17
Y	US, A, 4,829,005 (FRIEDMAN ET AL) 09 May 1989, see entire document.	18
Y	Clinical Microbiology And Infectious Disease, Volume 101, Number 1, issued January 1994, Park et al, "Rapid Diagnosis of Enterohemorrhagic Escherichia coli 0157:H7 Directly From Fecal Specimens Using Immunofluorescence Stain", pages 91-94, see entire document.	19 and 20
Y, P	Applied and Environmental Microbiology, Volume 60, Number 10, issued October 1994, Tortorello et al, "Antibody-Direct Epifluorescent Fliter Technique for Rapid, Direct Enumeration of Escherichia coli 0157:H7 in Beef", pages 3553-3559, see entire document.	19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05971

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Applied and Environmental Microbiology, Volume 59, Number 6, issued June 1993, Sharma et al, "Physical Characterization and Quantification of Bacteria by Sedimentation Field-Flow Fractionation", pages 1864-1875, see entire document.	18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05971

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 16/00, 17/00, 17/14; C12N 11/00, 11/14; C12Q 1/02; G01N 33/53, 33/533, 33/567

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